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ERRATA

"Volume 87, page 93, Figure 1. Invert figure to place the longer at the bottom with the vertical white lines for both descending from the top."

"Volume 87, page 125, Table I. In the third column headed *2 μgm. acetylcholine bromide* the bracket including compounds 4 to 10 should be divided into two brackets, one including compounds 4 to 8, the other compounds 9 and 10."

ALTERATIONS IN REPRODUCTIVE FUNCTIONS OF WHITE RATS ASSOCIATED WITH DAILY EXPOSURE TO NICOTINE[1]

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Most clinical and experimental reports favor the conclusion that chronic tobacco or nicotine poisoning is detrimental to reproductive processes. There are a few investigations, however, which contradict this conclusion. Critical examinations of the papers available reveals that many of the studies were poorly controlled or were limited to too few cases to permit statistical evaluation because of marked individual variations. The experiments reported herewith were planned in an attempt to obtain more dependable data bearing on this subject by using enough animals (3848) and proper design of experiments to permit statistical analysis. These experiments clearly show that nicotine interferes with the reproductive process.

REVIEW OF LITERATURE. *Fertility.* Support for a current opinion that tobacco has an effect upon the reproductive process in human beings is limited to statistical studies among women working in the tobacco industry (1, 2, 3, 4), to general observation (5, 6, 7, 8), and to isolated case reports (9). It would seem that social and economic conditions were factors of at least as much importance as tobacco in reducing the fertility of the workers. However, Phillips (9) established a correlation between smoking and immotility of sperm in a young man; cessation of smoking was followed by appearance of motile sperm. Resumption of smoking was followed by immotility of sperm and again cessation was followed by return of motile sperm.

Administration of tobacco, tobacco smoke, or nicotine to experimental animals was associated with abortions, permature births and stillbirths (10, 11, 12, 13, 14), decreased number of young born (13, 15, 16, 17, 18) or complete sterility (13, 19). One experimenter (Nice, 20) reported an increased fertility on the basis of results from groups of 3 to 6 animals.

Estrus cycle. Small but toxic doses had no effect on the estrus cycle (21, 22, 23, 24)[4] but larger doses decreased or abolished estrus (19, 25). Wilson and De Eds (26) noted a decrease in frequency of estrus of rats fed nicotine in various

[1] A preliminary report appeared in the J. Pharmacol. and Exp. Therap., 60: 100 (Proc), 1937.

[2] With the technical assistance of Florence Buel, Walter Buel, Carl Haase, Clenton Holt, Robert Sedgewick, and George Tanbara, appointees of the National Youth Administration, and of Grace E. Mascher. Supported in part by W P A Project number 165-1-07-234.

[3] Part of this report is taken from a thesis presented by Charles F. Lombard to the Graduate School in partial fulfillment of the requirements for the Ph D. degree.

[4] A typographical error appears on p. 114 of the paper by Behrend and Thienes (22). The maximum dose was 0.05 cc. of the 1 per cent solution, rather than 0 5 cc as printed in the report.

forms, but control experiments indicated that decrease in frequency of estrus was due to decreased food consumption.

Pathological changes in genital tract. The only statement found relative to the human ovary is that of Sajous (27) that the ovary "of the female habitué shrivels into a small kernel, hard and yellow." In experimental animals, atrophy of the ovary (19, 28) and of the testis (24, 29, 30) have been noted. Staemmler, who observed atrophy of the testis (29), could recognize no injury to the ovary (31). Inflammatory and degenerative change in the female reproductive tract have been reported (15, 32).

EXPERIMENTAL. *Methods.* Three separate experiments were performed. Procedures common to all the experiments will be described first, followed by special procedures for each experiment.

General procedures. Albino rats from the departmental inbred colony were used. These were kept in a room with temperature thermostatically controlled at from 26 to 28 degrees C., except on especially warm days when the temperature sometimes rose to 30 degrees. Each cage contained one control pair and one test (nicotine) pair, separated by a hardware cloth partition. Tap water and food were available to the rats throughout 24 hours.

The standard diet had the following formula: yellow corn meal 40 parts, ground whole wheat 28 parts, dry skim milk 18 parts, alfalfa meal 4 parts, linseed meal 6 parts, cod liver oil 2 parts, dried brewer's yeast 1 part, fish meal 1 part, sodium chloride 0.5 part, and calcium carbonate 0.5 part by weight. The standard diet was supplemented with twice weekly feedings of about 10 grams of fresh liver per adult rat. A few leaves of lettuce or other green food were placed in the cages twice weekly.

Control rats received early morning and late afternoon subcutaneous injections of 0.9 per cent sodium chloride solution and test rats received subcutaneous injections of varying dilutions of nicotine base in 0.9 per cent sodium chloride solution. The site of injection was rotated.

Records of weights of all rats were made weekly as well as the weights and number of each sex of young at birth and at weaning. The young were weaned one calendar month after birth.

Special procedures for each experiment. Experiment 1. On September 29, 1936 a large number of control and test male and female rats of approximately 60 days of age were selected. The sexes were kept separate and injected with 0.25 cc. per 100 grams body weight of saline (controls) or of 0.1 per cent nicotine in saline (test animals) respectively, subcutaneously, twice daily. In this experiment, an attempt was made to keep the nicotine dosage just below the convulsive dose, according to the susceptibility of the individual rats. The dose varied between 1.5 and 2.5 mg. per kilo. Tolerance development was rapid, so that most rats received the higher dose after one month.

On October 18, 1936, 25 control pairs and 23 test (nicotine) pairs from this group of rats were mated and injections continued. For this experiment, litter mates were not used, but control and test pairs were matched as closely as possible on the basis of body weight. Male and female were kept together throughout the experiment, in cages with $\frac{1}{2}$ inch mesh hardware cloth floors. The partitions separating the control from the test pairs was of $\frac{1}{4}$ inch hardware cloth. A few days before anticipated birth of young, metal nesting boxes 6 x $4\frac{1}{2}$ x 2 inches in size containing shavings were placed in the cages. Baby rats occasion-

ally escaped from these nests and fell through the hardcloth floors into the pan of sawdust below. A number died from exposure when this occurred at night, and were found and a record made in the morning.

Shortly after injections were started the rats began to die from bronchopneumonia and examination showed this to be associated with an infestation of mites, many of which had migrated into the lungs. Two treatments of the rats, a week apart, with a bath of S.A.E. 20 lubricating oil, and scrupulous care of the rats kept them free of mites subsequently.

Between March 13 and March 30, 1937, the males were killed with ether and various organs were removed for study. Injection of the females was continued until all pregnant animals had delivered and weaned their young. As the estrus cycle was re-established, the females were killed during anestrus and organs obtained for study. The data obtained from organ studies will be reported separately in a future communication.

Experiment 2. Because of the difficulties encountered in Experiment 1, due to the mite infestation and the loss of young through the hardware cloth cage floors, the experiment was repeated and extended with the following modification of method. A commercial fly spray, containing pyrethrins and organic isothiocyanates in petroleum distillate, was sprayed upon the cages after cleaning and upon the rats after returning to the cleaned and sprayed cages at weekly intervals. This practically eliminated mites from the colony and the incidence of pneumonia was greatly reduced.[5] To avoid accidental loss of young, the hardware cloth bottoms of the cages were removed and the rats were nested in shavings in removable pans at the bottoms of the cages.

Following June 14, 1938, adult test rats were injected with 0.1 cc. of 1:500 solution of nicotine in physiological saline per 100 grams body weight twice daily and litter mate controls were injected with corresponding volumes of saline solution. Since in this experiment it was desired to study effects of nicotine on succeeding generations, each litter born of these parent rats was divided as evenly as possible into two groups of each sex at about the age of 10 days, at which time there was enough hair to permit marking each baby rat with picric acid solution. The young were then injected with either nicotine solution or saline alone. These receiving nicotine were painted with picric acid over the caudal portion of the back and the control young were painted on the neck. The nicotine dose for the young was 0.001 cc. of 1:1000 nicotine solution per gram body weight and corresponding volumes of saline solution were injected into the controls. Young rats were weaned at the age of 4 weeks and injections were continued. The dose was increased 50 per cent when the young rats grew to 100 grams body weight and again increased to the adult dose of 0.1 cc. of 1:500 solution per 100 grams when a weight of 150 grams was attained.

At the age of approximately three months, the brother and sister, or cousin, male and female young were placed in cages for mating and injection, during October and November, 1938. Controls and nicotine injected pairs were litter

[5] A more extensive report of the use of fly spray for protection of rats is to be published later

mates. There were four groups of these second generation rats, as follows: (1) nicotine injected young from nicotine injected parents, designated as group N.N.; (2) nicotine injected young from control parents, designated as group C.N.; (3) control young from control parents, designated as group C.C.; and(4) control young from nicotine injected parents, designated as group N.C. Thus, in the symbols for the second generation groups the first letter refers to the parent group and the second letter to the second generation group. There were 20 pairs of rats in each of the second generation groups. An early death of a rat of the N.N. group due to accidental overdose with nicotine, reduce the number of pairs to 19. This experiment continued for a period of 10 months.

TABLE 1

Fertility of control and nicotine injected rats

	LINE		CONTROLS			NICOTINE INJECTED		
			Total pairs	Non-fertile pairs	Per cent non-fertile	Total pairs	Non fertile pairs	Per cent non-fertile
Experiment 1	1	0-59 days	4	3	75.00	9	4	33.33
	2	60-179 days	26	2	7.69	24	4	16.66
Experiment 2	3	CC	20	2	10 00			
	4	CN				20	7	35.00
	5	NC	20	5	25 00			
	6	NN				19	5	26.31
Experiment 3	7		46	12	26 08	39	17	43.59
Grand total	8		116	24	20.69	111	37	33.33
Grand total less line 1	9		112	21	18 75	102	33	32.35
Grand total less lines 1, 5, and 6	10		92	16	17 39	83	28	33.73

Experiment 3. For this experiment, litter mates were used for control and test groups, and brother-sister or cousin matings were employed. There were 46 control pairs and 39 nicotine-injected pairs. The experiment differed from experiment 2 in three important variables: (1) no diet supplement of liver and greens was given; (2) injections were given over a period of one year (Oct. 4, 1941 to Oct. 4, 1942): and (3) no injections were given during lactation, in order to control a possible factor in mortality of young, to be reported on in a subsequent paper.

RESULTS. *Fertile vs. non-fertile pairs.* Table 1 summarizes data on number of pairs found to have one or more litters as compared with pairs from which no litters were found. It is apparent from line 8 of the table that only 20.69 per cent of all control rats were non-fertile whereas 33.33 per cent of nicotine treated rats failed to bear young.

Elimination of data from parent rats which died during the first two months of experiment 1 (line 1, table 1) resulted in even more striking differences. Line 9 shows that there were 18.75 per cent non-fertile control pairs and 32.35 per cent non-fertile nicotine-injected pairs among the animals tested for more than 60 days. That these differences are statistically significant is indicated by a Chi square value of 19.5 when calculated from the formula

$$X^2 = \frac{(ad)^2\ (a + b + c + d)}{(a + b)\ (c + d)\ (b + d)}$$

where a, b, c, and d represent the figures shown in table 2. Since a Chi square value of 6 indicates not more than one chance in 100 that the differences

TABLE 2

Data of line 9, table 1 arranged for calculation of Chi square

TREATMENT	FERTILE PAIRS	NON FERTILE PAIRS	TOTAL
Controls	(*a*) 91	(*b*) 21	112
Nicotine	(*c*) 69	(*d*) 33	102
Total	160	54	214

TABLE 3

Re-arrangement of table 2 for calculation of $X^2 = \Sigma \frac{(A - T)^2}{T}$

A	T	$A - T$	$\frac{(A-T)^2}{T}$
a 91	83 7	7 3	63
b 21	28 3	−7 3	1.86
c 69	75 6	−6 6	58
d 33	25 4	7 6	2 86

$$\Sigma \frac{(A - T)^2}{T} = 5\ 93$$

are due to random sampling, the value of 19.5 indicates a very high probability of the differences being significant (33, 34). Using table 3, Chi square is calculated by the formula $X^2 = \Sigma \frac{(A-T)^2}{T}$ by the method suggested by Mainland (35) and a value of 5.93 is obtained; this represents a probability of between 0 02 and 0.01, which is well within the range of accepted statistical significance.

Elimination of second generation rats (NC and NN, lines 5 and 6, in table 1) from nicotine-injected parents further increases the differences in non-fertility between control and nicotine-injected animals (17.39 per cent *vs.* 33.79 per cent).

Number of litters. Table 4 summarizes the data on the influence of nicotine poisoning on the fertility of rats as measured by the numbers of litters born. In order to compare the different experiments, each of which was of different dura-

tion, the "litter index" for each group of rats was calculated. To make this calculation, the number of rat days was first determined by adding together the number of days during which the individual rats of each group were exposed to the experimental conditions. The litter index then is the quotient of the number of litters divided by the total number of rat days.

In Experiment 1, there seems to have been no influence of nicotine on the litter index. Discarding figures from rats which died during the first 2 months of the experiment, had no significant effect on the ratios. However, in Experiments 2 and 3, which were more extensive, both as to number of rats and as to the duration of the experiment, the litter index of the nicotine treated rats was approximately two-thirds that of the controls.

The figures for the second generation rats in Experiment 2 indicate an effect of nicotine received from the mother rats during gestation and the nursing period. Rats (NN and NC) from nicotine treated parents had fewer litters than rats (CC)

TABLE 4

Relation of chronic nicotine poisoning of white rats to number of litters born

EXPERIMENT		CONTROL RATS				NICOTINE-POISONED RATS			
		Number of pairs of parent rats	Number of litters	Total rat days	Litter index	Number of pairs of parent rats	Number of litters	Total rat days	Litter index
1		30	49	3430	.014	31	42	2993	.014
2	CC	20	68	5417	.013				
	CN					20	28	5187	.0054
	NC	20	48	5524	.0087				
	NN					19	44	5126	.0086
3		50	171	18,190	.0094	49	97	17,395	.0056

from control parents. There seemed, however, to be no significant difference between nicotine-injected rats (NN) of nicotine-injected parents as compared with control rats (NC) from nicotine-injected parents.

Further evidence for development of nicotine-resistance of progeny during nicotine injections in parent rats is the litter index (0.0086) for nicotine-injected rats (NN) from nicotine-injected parents which is higher than the litter index (0.0054) for nicotine-injected rats (CN) from control parents.

In Experiment 3, the relations are very similar to those of the corresponding groups (CC and CN) of Experiment 2.

In table 5, the litter index was calculated for only those rats which had one or more litters. Non-fertile rats, therefore, were not considered in these calculations, in order to determine effects of nicotine on rats known to be capable of bearing young. Furthermore, data from rats which died during the first two months of Experiment 1 are not included in table 5. Table 5 indicates a detrimental effect of nicotine upon the litter index of fertile rats not only of Experi-

ments 2 and 3, but also of Experiment 1. It further demonstrates nicotine tolerance in second generation rats.

The statistical analysis of the data indicates a high degree of probable significance of the differences between control and nicotine injected rats. The results of the statistical calculations are shown in table 8. The method of calculating "t" and "P" is that described by Fisher (34). For this purpose, the litter indices

TABLE 5

Number of litters born to control and to nicotine-poisoned fertile white rats

EXPERIMENT		CONTROL RATS				NICOTINE-POISONED RATS			
		Number of pairs of parents	Number of litters	Total rat days	Litter index	Number of pairs of parents	Number of litters	Total rat days	Litter index
1		23	48	2921	.0163	18	37	2132	.0126
2	CC	18	68	4857	.0140				
	CN					14	28	3731	.0075
	NC	15	48	4158	.0110				
	NN					14	44	3831	.0115
3		37	171	13,445	.0127	29	97	9969	.0097

TABLE 6

Number of progeny of control and nicotine-poisoned white rats

EXPERIMENT		CONTROL RATS				NICOTINE-POISONED RATS			
		Number of pairs of parent rats	Number of young	Total rat days	Progeny index	Number of pairs of parent rats	Number of young	Total rat days	Progeny index
1		26	307	2921	0.103	22	258	2132	0.121
2	CC	20	467	5417	0.086				
	CN					20	195	5187	0.038
	NC	20	284	5524	0.051				
	NN					19	257	5126	0.050
3		50	1108	18,190	0.061	49	640	17,395	0.037

for each pair of rats, rather than the indices calculated for the total group supplied the data for the calculations. Any value for P greater than 0.5, indicating one chance of error due to sampling in 20 samples, is of doubtful significance.

Numbers of young born. The "progeny indices" of control and nicotine poisoned rats of each experiment were calculated by dividing the total number of young born by the total number of rat days for each group in each of the three experiments. Table 6 gives the figures calculated from all rats (fertile and nonfertile) and table 7 for only the fertile rats. The trends are the same in each

table. "t" and "P" values are given in table 8. It is apparent that the progeny indices demonstrate about the same relation of nicotine poisoning to fertility as do the litter indices of tables 4 and 5. This statement applies to both first and second generation rats.

DISCUSSION. That chronic exposure to nicotine is associated with a decrease in reproduction seems clear from these experiments. The dose was relatively large, since it frequently caused convulsions in some of the rats with death of a few, and was therefore scarcely comparable to amounts which would be regularly absorbed from the use of tobacco in smoking or chewing or as an insecticide. Lombard (36) has pointed out that the relative daily dose injected into rats in these experiments would correspond to the maximum amount of nicotine which could be absorbed by a human subject from 40 average cigarets during smoking, assuming the same sensitivity in rat and man. However, it is possible that man is more sensitive to the toxic effects of nicotine than is the rat; in view

TABLE 7

Numbers of progeny of control and nicotine-poisoned fertile white rats

EXPERIMENT		CONTROL RATS				NICOTINE-POISONED RATS			
		Number of pairs of parent rats	Number of young	Total rat days	Progeny index	Number of pairs of parent rats	Number of young	Total rat days	Progeny index
1		23	307	2921	0.105	18	258	2132	0.121
2	CC	18	467	4857	0.096				
	CN					14	195	3731	0.052
	NC	15	284	4158	0.068				
	NN					14	257	3831	0.067
3		37	1109	13,445	0.082	29	640	9969	0.064

of the case reported by Phillips (9), our results are suggestive of possible harm to reproductive function in the human being, but at present this question cannot be confidently answered.

Experiments with rats (NC group) whose parents had been chronically poisoned with nicotine strongly suggest that exposure of rats to nicotine during pregnancy and nursing decreases the fertility of the young. This decreased fertility is exhibited not only by a larger number of which fail to bear young, but also by the decreased number of litters and of young born to the rats which were able to reproduce. Furthermore, when litter mates (NN group) of the rats of the NC group were injected with nicotine, there was no apparent further decrease in fertility by any of the criteria used, yet both the NC and NN rats were more fertile than first generation nicotine-injected rats (CN group). This result suggests that chronic nicotine poisoning of the parent rats not only decreases the fertility of their progeny, but also renders the progeny tolerant to the antifertility effects of subsequent poisoning with nicotine.

Another interesting observation is that discontinuous periods of injections of nicotine (Experiment 3) were associated with about the same degree of infertility as were the continuous periods (Experiments 1 and 2). At first thought, this might suggest that the effects produced by long continued dosage are due to the immediate pharmacological action of large doses rather than to chronic effects in the usual sense. If one can discriminate between effects of repeated dosage and chronic effects, we are inclined to think in terms of the latter, because of the results obtained with second generation rats.

TABLE 8

Statistical evaluation of tables 4, 5, 6, 7

GROUPS COMPARED	TABLE 4		TABLE 5		TABLE 6		TABLE 7	
	"t" value	"P" value	"t" value	"P" value	"t" value	"P" value	"t" value	"P" value
Exp. 1—C vs. N			0.06	0.90–1.00			1.01	0.30–0.40
Exp. 2—CC vs. CN	3.51	0.00–0.01*	2.89	0.00–0.01*	2.6	0.01–0.02*	2.06	0.02–0.05*
CC vs. NN	1.203	0.2 –0.3	.97	.3	2.3	0.02–0.05*	1.93	0.05*
CN vs. NN	0.72	0.4 –0.5	1.43	0.1 –0.2	0.36	0.7 –0.8	0.49	0.6 –0.7
NC vs. NN	0.42	0.6 –0.7	.145	0 9 –1	0.1	0 9 –1.0	0.32	0.7 –0.8
CC vs. NC	2.56	0.01–0.02*	1.01	0.3 –0.4	2.4	0.01–0.02*	2.08	0.02–0.05*
Exp. 3—C vs. N	2.90	0.00–0.01*	2.87	0.00–0.01*	2.93	0.00–0.01*	1.9	0.05*

Values of "P" marked with a star (*) are small enough to indicate that the differences between the indices of the two respective groups of rats are probably due to the experimental conditions rather than to errors of random sampling.

SUMMARY

Three separate experiments were made to determine the effect of repeated injections of nicotine upon reproduction of white rats. The experiments ran for 6, 10, and 12 months, respectively. Nicotine in subconvulsive doses (1 to 2 mg. per kilogram, in 10^{-3} to 2×10^{-3} concentration in physiological saline) was injected subcutaneously, twice daily. Controls received equal volumes of physiological saline. A total of 232 (116 pairs) control parent rats, 200 (100 pairs) nicotine poisoned parent rats, 2066 young rats from control parents, and 1350 young rats from poisoned parents served as a basis for statistical calculations.

Chronic nicotine poisoning was associated with an increase in the number of non-fertile pairs, and a decrease in the number of litters and of young born.

Progeny of chronically poisoned parents were less fertile than those of non-poisoned parents, but the fertility of the former was not further decreased by further exposure to nicotine.

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TOXICOLOGY OF 1,2-DICHLOROPROPANE (PROPYLENE DICHLORIDE)

II. Influence of Dietary Factors on The Toxicity of Dichloropropane

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1,2-Dichloropropane, $CH_2Cl \cdot CHCl \cdot CH_3$, is a colorless liquid with a density of 1.159 20°/20°C. and a boiling point of 96.8°C. It is finding extensive use as a solvent and as an insecticide. An investigation carried out in this laboratory indicates that the compound is one of the more toxic of the halogenated hydrocarbons (1).

This paper is concerned with the influence of certain dietary factors on the toxicity of dichloropropane for young rats. A comparable study of dichloroethane ($CH_2Cl \cdot CH_2Cl$), with references to the literature, has already been published (2).

Experimental. Groups of weanling rats were prepared for several weeks on diets which differed in their content of casein, fat, choline, l-cystine and dl-methionine Feeding was *ad libitum* except in experiments 6 and 7, in which the control rats were pair fed isocalorically against the low casein-high fat group. The rats were then given repeated 7-hour inhalation exposures to 1,000 parts per million (p.p.m.) of dichloropropane. No exposures were given on Saturdays and Sundays. The method of carrying out the inhalation exposures has been described previously (1) (2) (3).

In some of the experiments the chloroform soluble material in aliquot portions of fresh liver was determined by the method of Artom and Fishman (4).

The dichloropropane was a commercial product. Precision distillation of samples from the two lots of material used was carried out in an analytical fractionating column. This had a packed section 4 feet long and ½ inch in diameter, the packing being single turn pyrex helices $\frac{3}{32}$ inch in diameter. Measurements of specific gravity and index of refraction were also made. The data are recorded elsewhere (1). It was evident that the solvent probably contained a fair percentage of other isomers. For this reason the material used in experiments 5, 6 and 7 was redistilled in a 6 foot fractionating column. The fraction distilling between 96°C. and 97°C. was collected As will be noted below, the results obtained with the repurified compound were the same as those gotten with the commercial solvent.

Diets. The composition of the diets was as follows: salt mixture,[1] 2 per cent; cod liver oil, 5 per cent; varied amounts of purified casein[2] and hydrogenated cottonseed oil[3]; cane sugar, to make 100 per cent. The content of casein of the control diet was 25 per cent and its fat content (cottonseed oil) was 8 per cent. The low casein-high fat diet contained 6 per cent of casein and 38 per cent of fat. The diets also contained, in mg./kg., thiamine hydrochloride, 10; nicotinic acid, 40; pyridoxine hydrochloride, 10; calcium pantothenate, 40; and riboflavine, 20. The control diet contained 0 3 per cent of choline chloride. In

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[1] Hubbell, Mendel and Wakeman (5).

[2] SMACO.

[3] CRISCO.

experiments 5, 6 and 7 each rat received 3 mg. α-tocopherol in 0.03 cc. ethyl laurate per week orally. Supplements of choline chloride, dl-methionine or l-cystine plus choline chloride were added as indicated below.

RESULTS. In a preliminary experiment 23 weanling rats of the Wistar strain were fed semi-purified diets whose composition is described elsewhere (2). After

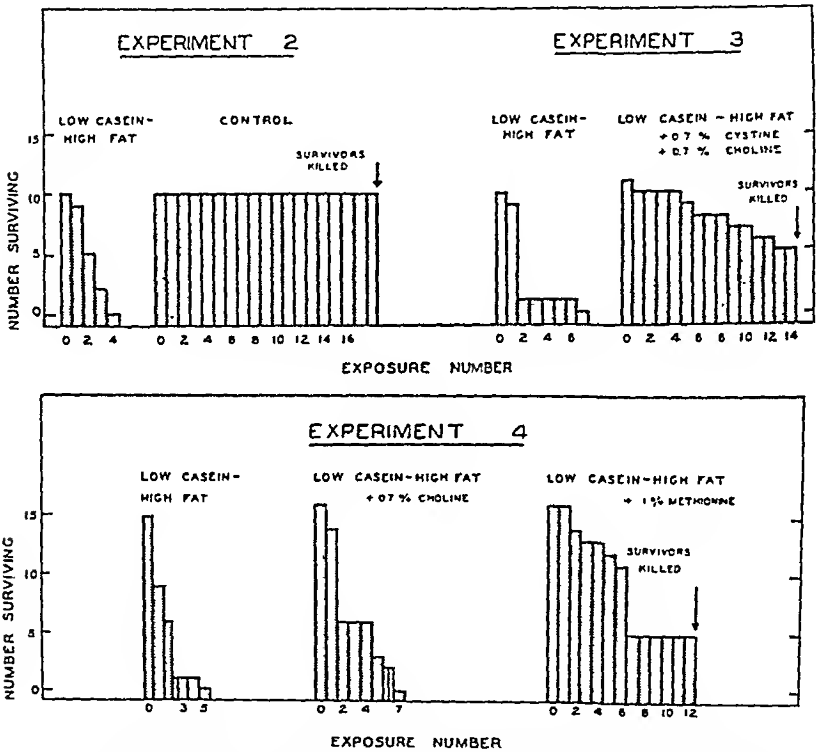

FIG. 1. Mortality among weanling rats with different dietary preparation and receiving repeated exposures to 1,000 p.p.m. of dichloropropane. The height of the first column of each graph indicates the number of rats placed on test. The height of adjacent columns indicates the number of rats surviving after successive exposures.

The period of dietary preparation was 21 days in experiments 2 and 3, and 23 days in experiment 4. In experiment 4 an observation period of 5 days intervened after the second exposure. Female Wistar rats were used in experiment 2 and Wistar rats of mixed sexes in experiment 3. Male rats of the Sprague-Dawley strain were used in experiment 4.

17 days on the diets exposures to dichloropropane were begun. The 11 rats on the low casein-high fat diet all died by the end of 3 exposures. Only 3 out of 12 control rats died, after 4, 5 and 15 exposures respectively. In this experiment a concentration of 1,500 p.p.m. of dichloropropane was used. When this was reduced to 1,000 p.p.m. and purified diets were used similar data were obtained (experiment 2, figure 1).

The low casein-high fat diet was deficient in both protein and choline. When 0.7 gm. of choline chloride was added to every 100 gm. of diet there was no important change in the mortality among young rats after exposures to dichloropropane (experiments 4, 6 and 7, figures 1 and 2). When supplements of 0.7 gm. of choline chloride and 0.7 gm. of l-cystine were used the animals became much more resistant to the effects of dichloropropane (experiments 3, 6 and 7, figures 1 and 2). Adding 1 gm. of dl-methionine to every 100 gm. of the low casein-high fat diet also afforded protection (experiments 4, 6 and 7, figures 1 and 2). The same relations obtained when a less severe choline deficiency was produced by feeding diets low in protein (6 per cent of casein) but normal in fat content (experiment 5, figure 2).

The great differences in mortality between the various groups were not related to differences in caloric intake. In experiments 6 and 7 (figure 2) the control rats were pair fed isocalorically against the low casein-high fat rats until most of the latter were dead. Furthermore, none of the supplements caused important increases in food intake or weight gain.

PATHOLOGICAL FINDINGS. Gross examinations were made on nearly all of the animals. Histopathological examination was carried out on 15 rats in experiment 1 receiving 1 or more 7-hour inhalation exposures to 1,500 p.p.m. of dichloropropane and on 10, 35, 29, and 60 rats in experiments 2, 4, 5 and 7 respectively, exposed similarly to 1,000 p.p.m. In addition examination was made of a large number of unexposed rats maintained on similar diets. Tissues were fixed in 10 per cent dilution of formalin and routine paraffin sections were stained with eosin-azure and with iron hematoxylin-picrofuchsin. Paraffin sections of liver and spleen were examined for hemosiderin. Paraffin sections of heart, liver and kidney were stained for fat by the method of Lillie and Ashburn (6).

Nearly all exposed and unexposed rats on the low casein-high fat diet showed markedly fatty livers characterized by large fat globules in the liver cells. Many showed traces of ceroid, chiefly in macrophages about the central veins of the liver. One rat on a low casein-high fat diet in experiment 2 showed renal scarring and pigmentation characteristic of the post nephrotic choline deficiency stage. Rats on low casein and low casein-high fat diets supplemented with choline, choline plus l-cystine, or dl-methionine showed slightly to markedly fatty livers characterized hy fine fat droplets in the liver cells. The amount of fat was more consistently large in exposed than in unexposed rats.

Certain infrequent lesions were seen only in exposed animals. In experiment 4, small areas of hemorrhagic necrosis were seen in the livers of 3 rats on a low casein-high fat diet supplemented with choline, 1 dying after 1, and 2 after 5 exposures to 1,000 p.p.m. of dichloropropane. Two other rats in experiment 4 on a low casein-high fat diet that died after 2 similar exposures showed extensive hemorrhagic necrosis of the adrenal cortex, and 1 rat in experiment 7 on a low casein-high fat diet supplemented by dl-methionine that died after 3 such exposures showed similar changes in the adrenal. Several animals on a low casein-high fat diet in experiment 2 that died after 2 to 3 exposures showed many scattered and occasional small masses of lipophages in the spleen.

More frequently occurring lesions seen only in the exposed animals were slight patchy or diffuse fatty degeneration of the heart and slight to marked fatty degeneration and necrosis of the kidney. A significant amount of fat was seen in 29 of 117 hearts stained for fat. Six of these 29 occurred among 10 rats on a low casein-high fat diet dying after 1 to 3 exposures to 1,500 p.p.m , 6 occurred in 32 rats on similar diets dying after 1 to 3 exposures to 1,000 p.p.m. and 6 in 16 rats on similar diets supplemented by choline dying after 1 to 3 exposures to 1,000 p.p m. Fatty degeneration of the kidney was characterized by the presence of numerous fine fat droplets in the epithelium of many tubules in the cortex. It was present in

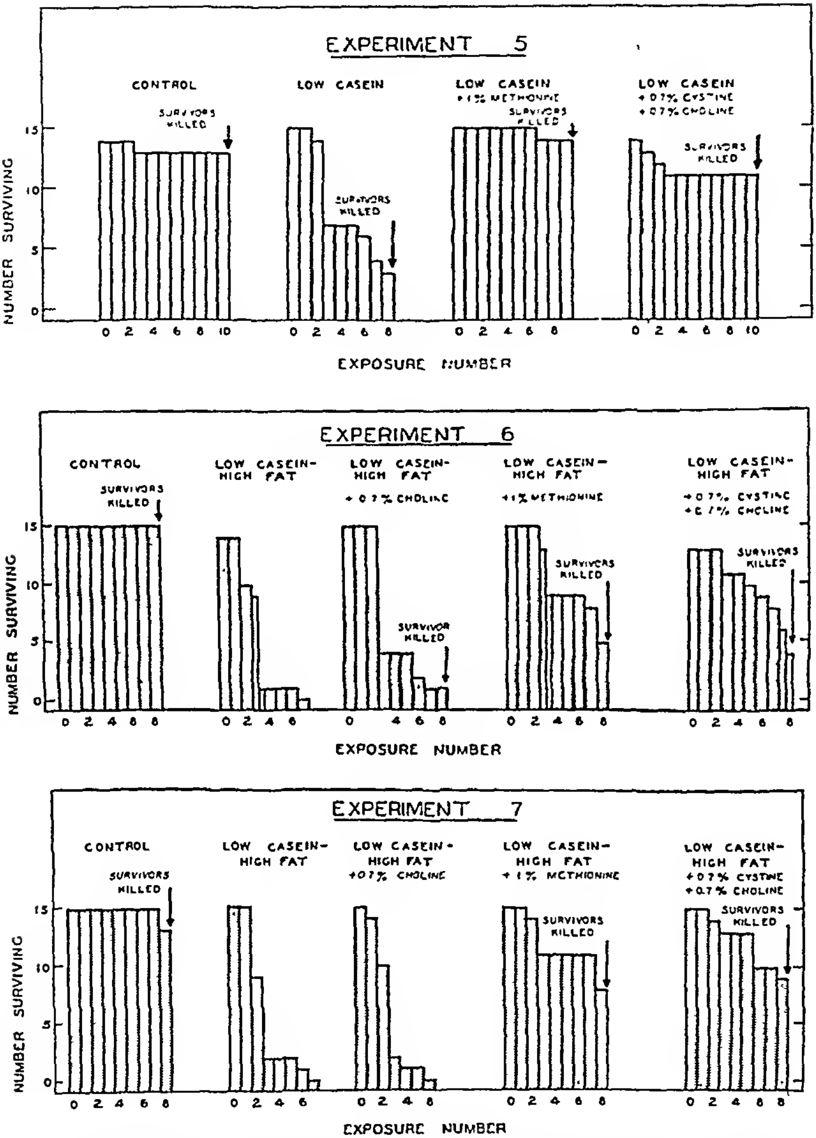

FIG. 2. Mortality among weanling rats with different dietary preparation and receiving repeated exposures to 1,000 p.p.m. of dichloropropane. The period of dietary preparation was 22 days in experiment 5, 21 days in experiment 6 and 26 days in experiment 7. Wistar rats of mixed sexes were used in experiment 5 and male rats of the Sprague-Dawley strain in experiments 6 and 7. An observation period of 8 days (experiments 5 and 6) and 5 days (experiment 7) intervened after the first 3 exposures.

TABLE 1

The liver fat content of weanling rats with different dietary preparation and which were exposed to dichloropropane vapors

DIET	DIETARY PREPARATION		LIVER FAT AS PER CENT OF FRESH TISSUE					
			Lived			Died		
	Av. daily food intake	Av. weight gain	No. of rats†	Mean	σ	No. of rats	Mean	σ
Experiment 4								
	gms.	*gms.*						
Low casein-high fat		−5.1				13.	38.5	5.3
Same, plus 0.7% choline		−6.2				13	8.0	2.4
Same, plus 1% methionine		−1.0	5	4.6	0.7	9	8.8	3.1
Experiment 5								
Low casein	3.1	0.5	3	6.5	1.4	9	12.4	5.4
Same, plus 1% methionine	3.3	4.3	10	6.8	0.9	1	9.2	
Same, plus 0.7% cystine and 0.7% choline	3.2	1.5	10	4.6	0.5	3	3.7	
Control		63.3						
Experiment 6								
Low casein-high fat	3.1	0.5				13	35.6‡	3.7
Same, plus 0.7% choline	3.1	0.7	1	4.1		10	8.3	2.5
Same, plus 1% methionine	3.1	4.5	5	7.0	2.0	8	11.1	2.0
Same, plus 0.7% cystine and 0.7% choline	3.0	3.9	4	5.1	0.5	9	9.1	3.9
Control	4.1*	28						
Experiment 7								
Low casein-high fat	2.5	−3.3				7	35.5	3.7
Same, plus 0.7% choline	2.5	0.3				11	13.8	4.4
Same, plus 1% methionine	3.2	4.9	6	4.8	0.3	3	11.3	0.7
Same, plus 0.7% cystine and 0.7% choline	3.0	3.6	7	4.5	0.6	1	11.3	
Control	3.3*	26.3	6	5.4	1.3			

* These amounts of food are isocalorically equivalent to what the low casein-high fat rats consumed.

† This refers to the number of rats whose livers were analyzed. The total number of rats involved in the experiments can be read from the charts (figures 1–3).

‡ The livers of 5 unexposed rats maintained on the low casein-high fat diet for about the same length of time were also analyzed. The mean fat content was 35.8 per cent with a standard deviation of 3.6 per cent.

nearly all rats that died after only 1 to 7 exposures to 1,000 p.p.m. or 1,500 p.p.m. and was usually minimal or absent in longer surviving killed animals and in unexposed controls.

Renal necrosis affected chiefly small groups of convoluted tubules. Severely affected tubules were converted into usually basophilic vacuolated fibrillar or granular masses;

less severely affected tubules showed a few marginal, frequently pyknotic or degenerated, nuclei and some oxyphilic shreds suggesting cellular remnants; and some tubules showed lesser changes grading into those of severe cloudy swelling or autolysis. In the more severely damaged kidneys the glomeruli were often shrunken, their nuclei pyknotic, and one or more lobules were occasionally necrotic. The glomerular spaces were often widened and sometimes contained some irregularly staining granular necrotic material occasionally admixed with some degenerating cells and nuclei. In less severely affected kidneys there was marked cloudy swelling with only an occasional necrotic tubule.

Because the presence and extent of renal necrosis was often obscured by marked autolysis, the frequency of renal necrosis in the rats that died can only be estimated. In experiment 1, 6 of 10 rats on a low casein-high fat diet that died after 1 to 3 exposures to 1,500 P.P.M. showed changes suggesting renal necrosis. In experiment 2, similar changes were seen in 5 of 10 rats on a low casein-high fat diet that died after 1 to 4 exposures to 1,000 P.P.M. In experiment 4, such changes were seen in 3 of 14 rats on a low casein-high fat diet that died after 1 to 2 similar exposures, in 4 of 13 rats on a similar diet supplemented by choline that died after 1 to 7 such exposures, and in none of 6 animals on a similar diet supplemented by dl-methionine that died after 3 to 7 such exposures. In experiment 7, changes suggesting necrosis were seen in 25 of 31 rats on various diets that died after 2 to 8 exposures to 1,000 P.P.M.; such changes were seen in each of 10 rats on a low casein-high fat diet and, in usually less severe form, in 7 of 10 such rats on a low casein-high fat diet supplemented by choline.

In killed animals, renal necrosis could be determined with ease. In experiment 4 it was seen in none of 6 animals on a low casein-high fat diet supplemented by dl-methionine that were killed after 12 exposures to 1,000 P.P.M. In experiment 5, rats were killed after surviving 9 to 10 exposures to 1,000 P.P.M.; renal necrosis was moderate to marked in all of 6 rats examined that were on a control diet containing 25% casein supplemented by 0.3% choline, but was absent in all of 13 rats on a low casein diet supplemented by l-cystine and choline and in all of 10 rats on a low casein diet supplemented by dl-methionine. In experiment 7, examination of rats killed after surviving 8 exposures to 1,000 P.P.M. showed slight to marked renal necrosis in 11 of 12 animals on the control diet, in 3 of 7 on a low casein-high fat diet supplemented by dl-methionine, in 4 of 8 on a similar diet supplemented by l-cystine and choline, and in 1 of 2 on a low casein-high fat diet supplemented by choline.

The relatively greater frequency and severity of renal necrosis in rats on low casein-high fat and control diets as compared with rats maintained on low casein and low casein-high fat diets supplemented by dl-methionine, choline, or choline plus l-cystine suggests that these supplements exert a strong protection against the nephrotoxic action of 1,2-dichloropropane beyond that due to the correction of a faulty diet. The nephrotoxic action of 1,2-dichloropropane on the control animals in experiments 5 and 7 was surprising in view of its absence in previous experiments with rats on comparable stock diets (1). Perhaps, the fact that the controls in these experiments were younger rats accounts for this. Also, in experiment 7, the control rats were underfed since their caloric intake was adjusted to that of the low casein-high fat rats.

Discussion. The data recorded here agree with the experimental results obtained in this laboratory with dichloroethane (2). Young rats prepared on low protein-choline deficient diets were more susceptible to the poisonous action of these halogenated hydrocarbons than rats on control diets. This increased susceptibility could be combatted by dietary supplements of dl-methionine or l-cystine plus choline chloride. Choline chloride alone was ineffective.[4]

[4] The published dietary studies of dichloroethane (2) indicated partial protection by choline. A number of experiments have since been carried out, in some of which partial protection by choline was obtained while in others there was no reduction in the toxicity of dichloroethane. Analysis of all of the data, involving several hundred rats, indicates no significant protection by choline.

Conceivably the choline deficient fatty liver might have taken up excessive amounts of these fat soluble hydrocarbons. But the negative results with supplements of choline chloride indicate that "fattyness" of the liver *per se* was not responsible for the high mortality among the low casein-high fat rats. It would appear that other factors are concerned which are more important than choline-deficiency and the resultant fatty infiltration of the liver. One of these appears to be the deficiency of sulfur containing amino acids, which may have some specific detoxifying function.

SUMMARY

(1) Weanling rats prepared for several weeks on low protein-choline deficient diets were more susceptible to the effects of inhalation exposures to 1,2-dichloropropane than were control rats on an adequate diet. This was true even in pair-feeding experiments.

(2) Supplements of choline did not appreciably increase the resistance of the choline deficient-protein deficient rats. dl-Methionine and the combination of l-cystine plus choline were quite effective.

(3) The pathological lesions found in exposed and control rats are described.

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2,2-BIS (p-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE (DDT) IN THE TISSUES OF THE RAT FOLLOWING ORAL INGESTION FOR PERIODS OF SIX MONTHS TO TWO YEARS[1]

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The demonstration of DDT in the tissues of rabbits (1) following relatively large acute oral doses posed the question whether the amounts found in the tissues represented actual storage, or were merely the result of temporary flooding of the organism. Definite indications that DDT is stored in the fatty tissues of dogs have been reported from this laboratory (2). We now report the results of studies on rats chronically exposed to DDT for periods of six months to two years.

Methods. Two experiments were conducted in which groups of young weanling male rats (21 days) were started on diets containing a commercial preparation of DDT composed of 81.8% p,p isomer and 18.2% o,p isomer. In the first experiment, two groups of 10 rats each were fed on diets containing respectively 800 and 1200 p.p.m. finely powdered dry DDT. The animls were maintained on a paired feeding regimen for six months, with an equal number of litter mates serving as controls for each group. Food consumption was adjusted daily, with the control receiving the amount of food its experimental mate had eaten on the previous day. In the second experiment 5 groups of 12 rats were fed on diets containing respectively 0, 100, 200, 400 and 800 p.p.m. DDT incorporated in corn oil solution. These rats were fed ad libitum and carried through for two years. At the termination of the experiments the surviving animals were killed by decapitation and exsanguinated. Livers, kidneys and other tissues were removed and weighed. The concentration of DDT in the tissues was determined by a bio-assay method (3) and the distribution of the o,p and p,p isomers of DDT in fatty tissue and diet was determined by the Schecter method (4). The oxygen consumption (QO_2) of slices of liver tissue, suspended in phosphate-buffered glucose-Ringer solution, was determined by the standard Warburg procedure. Readings were begun within one hour after removal of the tissue from the animal, and repeated at 30-minute intervals for a period of three hours.

Results. *Paired feeding experiment, six-month exposure.* All of the animals, whether on 800 or 1200 p.p.m. DDT, showed characteristic symptoms of DDT poisoning. During the experimental period 5 rats on 800 p.p.m. DDT and 3 rats on 1200 p.p.m. DDT died. These animals showed extreme tremors and convulsions before death. Those that survived, exhibited only moderate tremors notably during the early part of the exposure and later appeared to recover. Table 1 shows that the experimental rats grew slower than their litter mate controls. Unless it was losing weight, or in extremis, the experimental animal ate more food than its litter mate control needed; in nearly every case

[1] The work described in this paper was done under a transfer of funds, recommended by the Committee on Medical Research, from the Office of Scientific Research and Development to the Food and Drug Administration.

there was extra food left over in the food cup of the control. Nevertheless, the control rat grew at a greater rate. To account for this observation, one may assume that the experimental animal in its excitable state dissipated more energy and hence required more food.

TABLE 1

Mean increase in weight of young male rats on a paired feeding regimen with DDT for 6 months

DOSAGE	NO. OF RATS	MEAN GROWTH	STANDARD ERROR OF MEAN	P
		Grams		
1200 p.p.m.	7	320.6	±9.91	<0.01
Control	7	373.8	±11.08	
800 p.p.m.	5	338.4	±7.73	<0.01
Control	5	387.2	±12.57	

TABLE 2

The effect of chronic ingestion of DDT on the weight of the liver and kidneys of individual rats

	GRAMS LIVER (LITTER MATE PAIRS)		GRAMS KIDNEYS (LITTER MATE PAIRS)	
	Experimental	Control	Experimental	Control
1200 p.p.m. DDT	22.2	15.9	3.1	2.6
	22.8	14.8	3.6	2.8
	22.8	15.9	2.9	2.5
	19.6	14.4	2.6	2.2
	19.1	13.4	2.7	2.6
Avg.	21.3	14.9	3.0	2.5
800 p.p.m. DDT	20.6	15.5	3.5	2.5
	25.3	15.4	3.4	2.3
	19.2	13.6	2.9	2.5
	24.5	18.2	2.9	3.1
	22.6	15.9	2.6	3.0
Avg.	22.4	15.7	3.1	2.7
Per cent increase over control	43		19	

At autopsy it was noted that the livers, and to a lesser extent the kidneys of the experimental animals, were larger than those of the litter mate controls. Weighing confirmed this observation, the results of which are shown in table 2. This increase in weight of the experimental over the control tissues could not be accounted for by hydration; there was actually more tissue present on the basis of dry weight. Pathological examination confirmed this, for there was found a moderate degree of centrolobular hypertrophy in the liver. The data in table 2 show that with the exception of 2 kidney pairs all of the experimental tissues

weighed heavier than their corresponding controls; but there appears to be no differentiation between the 1200 and 800 p.p.m. levels of DDT. Finally, the hypertrophic effects of DDT become even more striking when it is remembered that the lighter organs came from the heavier control animals, and the heavier organs from the lighter experimental animals.

In table 3 are presented the results of the tissue respiration study. Without exception the QO_2 is notably lower in the experimental tissues—averaging 40% below the control values, and here also there appears to be no differentiation between the 1200 and 800 p.p.m. levels of DDT. Since the QO_2 for each tissue represents the average value over a 3 hour observation period, it should be added

TABLE 3

The effect of chronic ingestion of DDT on the respiration of liver slices from individual rats

	MICROLITERS O_2 PER MGM. DRY TISSUE PER HOUR (QO_2)* (LITTER MATE PAIRS)	
	Experimental	Control
1200 p.p.m. DDT	5.8	8.9
	6.2	8.6
	5.6	9.7
	4.4	9.0
	6.7	11.3
Avg.	5.7	9.5
800 p.p.m. DDT	7.4	11.1
	3.9	7.4
	5.1	8.9
	5.3	8.0
	4.5	9.4
Avg.	5.2	9.9
Per cent decrease under control	40	

* Average over a three-hour period.

that not only were all the individual experimental QO_2 values, read at half-hourly intervals, lower than the controls, but plotting these values indicated that the slopes of the experimental and control QO_2—time curves were essentially parallel, a condition which meant that the decrement rates of oxygen consumption were the same. The lowered QO_2 of the chronically poisoned liver tissue may perhaps be expected in view of the evidence of moderate hypertrophic changes which have been shown to occur.

In table 4 are given the results of the analyses of rat tissues for DDT by the bio-assay method. With the exception of two kidneys, measurable amounts of DDT were found in all tissues examined. No DDT was found in any control tissues. As noted with some of the other effects of DDT, no correlations between concentration of this substance in tissues and in diet can be detected, but this

observation is restricted to the high levels only (1200 and 800 p.p.m. DDT in diet). To be emphasized particularly is the fact that the amount of DDT in the perirenal fat is roughly 50 to 100 times as great as in any of the other tissues. The variation in the DDT concentration in liver, spleen and muscle, together with the limited number of analyses, makes it doubtful whether any of the differences are significant. It would appear that the DDT concentrations in spleen and muscle tend to be higher than in liver and kidney, but only between muscle and liver can a statistically significant difference ($p = 0.04$) be demonstrated. Because of the apparent affinity of DDT for fatty tissue, the question arises whether the presence of fat in such tissues as liver, kidney and spleen might account for the DDT found therein. Gravimetric determinations of ether-soluble material extracted from these tissues show that the "fat" content

TABLE 4

The DDT content of the tissues of individual rats following ingestion of DDT for a period of 6 months

	PERIRENAL FAT	DDT CONTENT OF FRESH TISSUE				
		Liver	Kidney	Spleen	Muscle	Brain
	mgm./gram	*micrograms per gram*				
1200 p.p.m. DDT	2.7			18	15	
	2.1		10	18	14	
	3.3	7.1	22	24	22	
	2.1	8.4	13	21	21	
	2.5	7.2	6.8	29	34	
800 p.p.m. DDT	2.4	5.4	9.6	13	30	
	2.7	3.8	6.8	10		
	2.9	17	17	8.7		
	2.8	22	?	14		29
	4.9	21	?	34		18

averages about 2%. On this basis, if the DDT found in liver, kidney and spleen was dissolved in the fatty components of these tissues, its concentration therein would range from 0.25 to 1.0 mgm. per gram. While this range of calculated values is somewhat less than the DDT content of perirenal tissue (95% fat), it is, nevertheless, roughly of the same order of magnitude. This would indicate that the DDT content of the liver, kidney and spleen could be explained on the basis of the fat content of these tissues. In the case of muscle, however, where care was taken to select only lean tissue, the fat content averaged only 1%, and yet the DDT content tended to be highest. It may be that some other mechanism is involved in the storage of DDT in this tissue.

In table 5 are given the results of the analyses of six samples of perirenal fat for DDT by the biological and chemical methods. In the case of the chemical method which is based on the development of a color, it is possible to determine the p,p and o,p isomers of DDT separately by making readings at 580 and 510

Mμ respectively. It can be seen that the biological and chemical methods are in essential agreement on the content of the p,p isomer of DDT. (The biological method does not determine o,p isomer). Since, however, the DDT in the diet consisted of a mixture of 81.8% p,p isomer and 18.2% o,p isomer, it became of interest to examine whether this ratio would also obtain for the DDT stored in

TABLE 5

The distribution of the p,p and o,p isomers of DDT in the perirenal fat of the rat

RAT NO	BIOLOGICAL ASSAY	CHEMICAL ASSAY			PROPORTION OF ISOMERS IN FAT	
		p,p isomer	o,p isomer	total	p,p isomer	o,p isomer
	Milligrams of DDT per gram of fat				Per cent	
1	2.8	3.0	0.9	3.9	76	24
2	4.9	3.9	0.8	4.7	83	17
3	2.1	2.2	0.3	2.5	86	14
4	3.3	3 2	1.1	4.3	76	24
5	2.1	2.5	0.6	3.1	80	20
6	2.7	3 2	0.7	3.9	83	17
Avg.	3.0	3.0			80.7	19.3
Analyses of sample DDT fed					81.8	18.2

TABLE 6

The DDT content of the tissues of individual rats following ingestion of DDT for a period of two years

DDT CONTENT OF DIET	DDT CONTENT OF FRESH TISSUE		
	Perirenal fat	Liver	Kidney
p.p m.	*mgm /gram*	*micrograms per gram*	
100	0 103		
100	0.129		
100	0.091		4.6
100	0.058		
200	0.313	3.1	14
200	0.158		
400	1.091	2.7	20
400	0.966		
800	4.220	23	64

the fat. That this does in fact occur is shown in table 5. Presumably the organism does not differentitate between the isomers when it comes to storage, but lays them down in fatty tissue in the same proportion in which they occur in the diet.

Two-year exposure to DDT. In table 6 are given the DDT analyses of tissues removed from rats exposed for two years. These rats represent the survivors of the original group of 12 animals placed on each level of DDT. Of the control

group of 12 animals there were 4 survivors. Detailed reports of the nutritional and pathological findings will be described elsewhere. In contrast to the six-month experiment at 800 and 12 p.p.m. DDT there is here a definite correlation between tissue level and dose level, and this is most clearly seen in the case of the perirenal fat. It is interesting to note that in the perirenal fat of the rat exposed for 2 years to 800 p.p.m., the DDT concentration is roughly of the same order of magnitude as that found in the fat of the animals exposed to 800 p.p.m. for only 6 months (table 4). On the other hand, if the kidneys are compared, it is seen that the longer exposure has produced 4 to 5 times as high a concentration of DDT.

SUMMARY

Rats were fed diets containing from 100 to 1200 p.p.m. DDT for periods ranging from six months to two years. The following effects were noted.

1. At 800 and 1200 p.p.m. DDT in the diets, rats showed characteristic symptoms of poisoning which terminated fatally in some cases. There was definite depression of growth.

2. At 800 and 1200 p.p.m. DDT in the diets rats showed an increase of 43% and 19% respectively in the weights of their livers and kidneys over those of the controls.

3. At 800 and 1200 p.p.m. DDT in the diets, the oxygen consumption of the livers was reduced 40% below that of the controls.

4. DDT was found in all tissues of animals exposed to DDT. Due apparently to its preferential solubility in fat, the concentration of DDT in perirenal fat was roughly 50 to 100 times as great as in other tissues. With the exception of muscle, it was indicated that the DDT of other tissues could be explained by their fat content.

5. As far as storage is concerned, the rat did not distinguish between p,p and o,p isomers of DDT, but laid these down in the same proportions at which they were fed in the diet.

6. The concentration of DDT in fatty tissue was found to be correlated with the level of DDT in the diet in the range of 100 to 800 p.p.m.

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ON THE EXPECTORANT ACTION OF PARASYMPATHOMIMETIC DRUGS[1]

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Parasympathomimetic drugs are not ordinarily considered as expectorants. If it be agreed that, amongst their various pharmacological reactions, expectorants have the property of augmenting the production or output of bronchial or respiratory tract fluid (R.T.F.), then our findings are that most parasympathomimetic drugs have marked expectorant properties. Indeed, our experiments have demonstrated that drugs in the parasympathomimetic group increase the output of R.T.F. to a much greater extent than occurs following presentation of the drugs commonly classified as expectorants in textbooks of pharmacology and therapeutics. Using the technique developed in this laboratory for quantitatively measuring the effect of drugs upon the output of R.T.F. in animals, it has been found that there is some variation from species to species, but an approximate comparison may be obtained from the following reports.

Perry and Boyd (1) found that ammonium chloride increased the rate of output of R.T.F. by a maximum of 88 per cent, ammonium carbonate by 110 per cent, powdered ipecac, B.P., by 143 per cent, glycerol guaiacolate by 185 per cent and preliminary experiments upon 3 cats showed that pilocarpine nitrate raised the output by over 600 per cent. Stevens et al. (2) reported guaiacol maximally increased the output of R.T.F. by 78 per cent, guaiacol carbonate by 60 per cent, creosote by 123 per cent and guaiacol glycerol ether by 121 per cent. Boyd, Jackson and Ronan (3) investigated the action of several sympathomimetic amines and found that adrenaline hydrochloride maximally increased the output of R.T.F. by a mean of some 50 per cent, ephedrine hydrochloride by 3 per cent, neo-synephrine hydrochloride by 2 per cent, privine hydrochloride by 37 per cent and amphetamine sulphate by 45 per cent. They also found that faradic stimulation of the cervical sympathetic trunk augmented the output of R.T.F. by 12 per cent whereas Perry and Boyd (1) have found that corresponding electrical stimulation of the cervical vagus trunk maximally increased the output by a mean of 200 to 300 per cent. Boyd and Munro (4) gave ether by inhalation and could detect no appreciable change in the output of R.T.F. in guinea pigs, rabbits and cats but there was a 300 per cent increase in dogs which was not affected by administration of atropine sulphate. Boyd, MacLachlan and Perry (5) performed corresponding experiments with inhalation of ammonia gas and got about a 150 per cent increase in the output of R.T.F. Boyd and MacLachlan (6) gave paregoric to albino rats, hens, guinea-pigs, rabbits and cats and obtained maxi-

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mal increases up to 400 per cent in the output of R.T.F. in albino rats; of the components of paregoric, tincture of opium produced a maximal rise of 58 per cent, camphor of 67 per cent and 60% alcohol of 88 per cent. Boyd et al. (7) demonstrated that potassium iodide increased the output of R.T.F. to a maximum of some 150 per cent, and also found the organic iodides, Siomine, N.N.R., and Iod-Ethamine or ethylenediamine dihydriodide (Pitman-Moore Co.) to augment the output some 100 per cent; Iodo-casein, N.N.R., Iodalbin, N.N.R., Iodostearine, N.N.R., Oridine, N.N.R., Stearodine, N.N.R., Lipoiodine, N.N.R., Sajodin, N.N.R., Lipiodol, N.N.R., and Iodicin (Burroughs Wellcome and Co.) had little or no effect upon the rate of output of R.T.F. Boyd and Pearson (8) studied the effect of volatile oils given by stomach tube and reported oil of eucalyptus augmented the output of R.T.F. to a maximum of 172 per cent, oil of lemon to 240 per cent, oil of anise to 552 per cent, oil of turpentine to 160 per cent, oil of pine to 103 per cent, terebene to 88 per cent, terpin hydrate to 96 per cent and Friar's Balsam had no appreciable effect. Boyd and Dorrance (9) were surprised to find that sulphanilamide augmented the output of R.T.F. to a maximum of 55 per cent, sulphathiazole to as high as 650 per cent, sulphadiazine to 216 per cent while sulphamerazine had no significant effect.

Thus it may be seen that most of the commonly used expectorants, which have been studied in this laboratory, augment the output of R.T.F. to a maximum of between 100 and 200 per cent. In contrast, we have encountered several parasympathomimetic drugs whch maximally increase the output of R.T.F. by from 1,000 to 1,500 per cent. These results suggest parasympathomimetic drugs would be excellent additions to cough mixtures but, of course, the stumbling block to such use is the unfortunate fact that parasympathomimetic drugs have many other pharmacological properties which might be undesirable in a cough medicine. It seems to us that this disadvantage could be surmounted and that synthetic modifications of parasympathomimetic drugs might yield one or more which would possess marked expectorant properties, and undesirable properties to little or no extent.

Cholinergic drugs and the volume output of R.T.F. The technique used for collecting R.T.F. in the experiments to be described was that of Perry and Boyd (1) as modified by Boyd, Jackson and Ronan (3). Between 350 and 400 animals were used, including over 300 cats and some dogs, rabbits, guinea-pigs and albino rats. The general procedure was to arrange the animals for collection of R.T.F. and, at the end of 3 hours, a stated dose of drug was given subcutaneously. To reduce the voluminous data to a common denominator for comparative purposes, the volume output of R.T.F. was taken hourly, expressed as ml. per kilo body weight per 24 hours, the values for each dose in each species averaged and then the increased output hourly after administering the drug expressed as a percentage of the average output of R.T.F. during the two hours just preceding giving of the drug. These means, as obtained in healthy, adult cats, are given in table 1.

For convenience, the various drugs used were divided into three groups, termed respectively the choline group, the physostigmine group and the pilocarpine group. To begin with, it may be noted that the drugs were dissolved in saline and in-

TABLE 1

The effect upon the rate of volume output of respiratory tract fluid in cats of a range of doses of parasympathomimetic drugs given subcutaneously

DRUG	DOSE	NO. OF ANIMALS	PER CENT INCREASE IN RATE OF OUTPUT OF R.T.F.			
			1st hr.	2nd hr.	3rd hr.	4th hr.
	mgm./kilo					
Saline	1 ml./kilo	10	−8	17	6	14
The choline group						
Acetylcholine	2	4	−31	−25	−55	−61
Acetylcholine	20	2	−60			
Acetylcholine	40	7	26	10	29	−6
Acetylcholine	100	3	Lethal			
Mecholyl	5	7	40	−13	−7	−7
Mecholyl	7.5	6	118	263	−36	0
Mecholyl	10.0	8	842	528	214	43
Carbachol	0.01	6	−40	−19	−14	−9
Carbachol	0.05	5	62	−5	10	5
Carbachol	0.1	7	780	1,420	Lethal	
Urecholine	0.5	13	231	135	26	−35
Urecholine	1.0	8	11	323	412	100
Furmethide	0.1	11	−55	−64	−36	41
Furmethide	0.3	6	4	18	111	141
Furmethide	0.5	4	67	Lethal		
Furmethide	1.0	4	128	Lethal		
The physostigmine group						
Physostigmine	0.01	10	10	10	33	−19
Physostigmine	0.1	7	0	4	11	−14
Physostigmine	0.5	8	−16	33	14	0
Physostigmine	0.75	9	31	−22	−19	−16
Physostigmine	1.0	3	Lethal			
Prostigmine	0.1	12	−54	−15	−46	−23
Prostigmine	0.2	11	−38	38	87	137
Prostigmine	0.5	5	83	0	Lethal	
Compound A	0.5	14	185	348	191	−71
Compound A	1.0	8	1,460	1,080	606	533
Compound B	1.0	10	13	21	43	−9
Compound B	5.0	14	470	300	Lethal	
The pilocarpine group						
Pilocarpine	0.05	8	12	12	−3	18
Pilocarpine	0.25	12	10	−10	50	−10
Pilocarpine	0.5	13	30	35	0	−50
Pilocarpine	1.0	8	156	72	13	−32
Arecoline	1.0	9	131	37	−3	−23

jected in a total volume of 1 ml. per kilo body weight. A control group of cats, given only this volume of saline, showed no appreciable change in the volume output of R.T.F.

In the choline group, acetylcholine bromide had no marked effect upon the volume output of R.T.F., as might be expected. We are at a loss to account for the apparent decrease in output of R.T.F. produced by the smaller doses of this and of some other parasympathomimetic drugs. In selecting the range of doses of acetylcholine bromide and most other drugs we began with small doses, of the order per unit body weight of those recommended for man, and worked up to the lethal dose. In contrast to the indifferent results with acetylcholine, the more stable derivatives, mecholyl chloride (acetyl-betamethylcholine chloride), carbachol (B.P. 1932, 3rd Addendum, 1941) or carbaminoycholine chloride and urecholine (betamethylcholine urethane), all kindly provided by Merck and Company, Limited, of Montreal, had a marked effect upon the volume ouput of R.T.F. as may be seen from the data summarized in table 1. The action of furmethide (furfuryl-trimethyl-ammonium iodide), kindly provided by Smith, Kline and French Laboratories, was definitely positive in the larger doses but not to the same degree as that of the three former drugs.

In the physostigmine group, physostigmine salicylate, B.P. had little effect upon the volume output of R.T.F. Prostigmine methyl sulphate, N.N.R., kindly provided by Hoffmann-La Roche Limited of Montreal, had an appreciable positive effect in the larger doses. In the pilocarpine group, both pilocarpine nitrate, B.P., and arecoline hydrochloride definitely increased the volume output of R.T.F. in the larger doses. Two new compounds, chemically related to physostigmine, were placed at our disposal by the research laboratories of Merck and Company. That labelled Compound A in table 1 is the dimethylurethane of m-isopropyl-p-dimethyl-aminophenol methiodide and that labelled Compound B is the dimethylurethane of p-dimethylamino thymol methiodide. These compounds were reported to have cholinergic activity by Stevens and Beutel (10). Both Compounds A and B were found to have a pronounced positive effect upon the rate of volume of output of R.T.F., the former moreso than the latter.

Species variation. Taking all factors into consideration, cats were found the animals most suitable for this work. However, it was decided to make certain that the results obtained in cats were not restricted to this species alone and a few experiments were performed upon albino rats, guinea-pigs, rabbits and dogs, using large doses of pilocarpine nitrate as an example of the parasympathomimetic drugs. Experiments performed upon five albino rats were unsatisfactory. In 18 guinea-pigs, the rate of output of R.T.F. was increased to a maximal amount of 64 per cent. In 14 rabbits, the maximal increase in output of R.T.F. averaged 362 per cent. In 10 dogs, the corresponding value was 502 per cent increase. These results indicate that pilocarpine nitrate, and probably also the other active parasympathomimetic drugs, increase the output of R.T.F. to a considerable degree in the species mentioned as well as in cats.

The effect of atropine sulphate. Atropine sulphate injected alone into rabbits, cats and dogs has little or no effect upon the volume output of R.T.F. (4). When given to animals with an augmented ouput of R.T.F. due to administration of parasympathomimetic it reduced toward normal this augmented output. This was proven in 42 cats given 1 or 2 mgm. per kilo body weight of pilocarpine nitrate with or without simultaneous injections of increasing doses of atropine

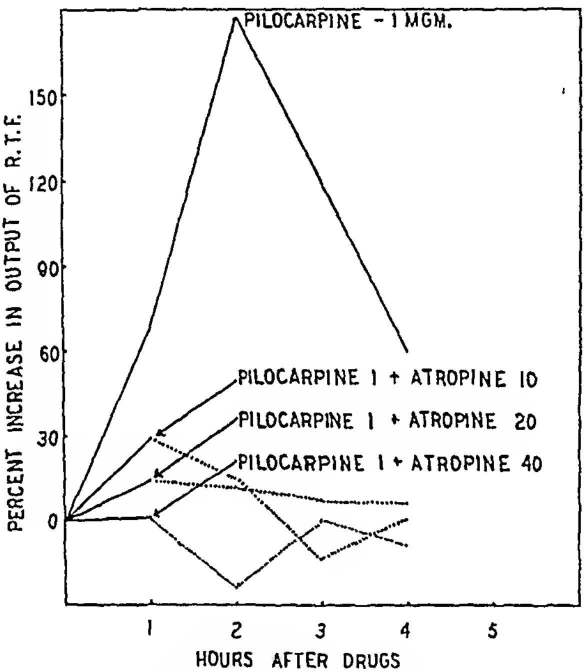

FIG. 1. THE PERCENTAGE INCREASE IN THE OUTPUT OF R.T.F. IN CATS PRODUCED BY INJECTION OF PILOCARPINE NITRATE AND THE EFFECT THEREON OF SIMULTANEOUS INJECTION OF INCREASING DOSES OF ATROPINE SULPHATE

sulphate. The results were averaged, calculated on the basis of percentage change in the volume output of R.T.F. as before and the mean data have been plotted in figure 1.

The administration of pilocarpine nitrate alone produced the usual augmented output of R.T.F. When atropine sulphate was given at the same time in doses of 10 mgm. per kilo body weight, the maximal increase in the output of R.T.F. was only 27 per cent. Increasing the dose of atropine sulphate to 20 mgm. per kilo body weight, reduced the mean maximal increase from pilocarpine to 14 per cent. The combination of pilocarpine nitrate with 40 mgm. per kilo of atropine sulphate completely eliminated the increased output of R.T.F. These results suggested that pilocarpine nitrate, and probably the other active cholinergic

drugs, increased the output of R.T.F. most likely by acting upon the cholinergic receptor cells in the secretory glands of the respiratory tract. The doses of atropine sulphate required to neutralize the expectorant effect of pilocarpine were larger than would be expected and this suggests the possibility that the glands of the lower respiratory tract are resistant to the action of atropine.

Section of the cervical vagus nerve. In further studies upon the mechanism of action of pilocarpine nitrate (again selected as an example of parasympathomimetic drugs) in augmenting the output of R.T.F., the vagus nerve was cut above its branches to the lungs in 17 cats. The animals were arranged for collection of R.T.F. and pilocarpine nitrate injected in a dose of 1 to 2 mgm. per kilo body weight. In all except one animal there followed an increase in the output of R.T.F. The mean percentage increases for each successive hour after injecting pilocarpine were: 1st hour—290 per cent, 2nd hour—179 per cent, 3rd hour—111 per cent and 4th hour—43 per cent.

These results prove that pilocarpine nitrate, and probably the other active parasympathomimetic drugs, increased the output of R.T.F. not by acting centrally but by acting directly upon the secretory cells of the respiratory tract. It is of interest that practically all expectorant drugs studied in this laboratory have been found to act reflexly from the stomach. One important exception was the group of expectorant volatile oils which were proven not to act reflexly from the stomach but probably directly upon the secretory cells of the respiratory tract (8). To this group may now be added the parasympathomimetic drugs.

Decerebrate cats. In all of the above experiments, the animals were lightly anaesthetized with intraperitoneal injections of urethane in a dose sufficient to produce about 25 per cent of surgical anaesthesia according to Guedel's criteria, i.e. to about the bottom of plane I of Stage 3. It was considered that the use of an anaesthetic agent might have depressed the expectorant action of parasympathomimetic drugs. Hence 11 cats were decerebrated, arranged for collection of R.T.F. and after 3 hours given a subcutaneous injection of 2 mgm. per kilo body weight of pilocarpine nitrate, which was selected again as an example of parasympathomimetic drugs. There followed an increase in the output of R.T.F. of about the same order of magnitude and duration as in the urethanized animals.

Seasonal variation. No systematic investigation was made of the effect of season upon parasympathomimetic drugs and R.T.F. production. It may be noted, however, that the original experiment upon pilocarpine nitrate was performed in the summer months and a considerable increase in the output of R.T.F. was obtained with a dose of 1 mgm. per kilo body weight. When this dose of pilocarpine was given later in the winter and spring, to our surprise it failed to have its usual effect. The sample of the drug was taken from the same bottle as had been used previously, so that the difference could not be ascribed to variations in the potency of batches of pilocarpine nitrate. Hence a series of experiments was performed upon cats, using a range of doses of pilocarpine nitrate. It was found that in the winter and spring months, approximately double the dose had to be used to increase the output of R.T.F. to the same extent as in the summer months.

The specific gravity of R.T.F. In the experiments described above, the sole effect of parasympathomimetic drugs discussed was their influence upon the volume output of R.T.F. In addition to investigating the volume output, we also studied changes, if any, in the specific gravity of R.T.F. Since the volume of R.T.F. produced per hour is small, the procedure followed was to measure the specific gravity of the entire output of R.T.F. during the three hours before giving a parasympathomimetic drug and repeat this for the four hours following

TABLE 2

The effect upon the specific gravity, relative viscosity and chloride content of respiratory tract fluid in cats of subcutaneous injection of a range of doses of parasympathomimetic drugs

DRUG	DOSE	SP. GRAVITY	RELATIVE VISCOSITY (DISTILLED WATER = 1,000)	CHLORIDE
	mgm./kilo			*mgm./100 ml.*
Saline, 1–3 hrs.	1 ml./kilo	0.9980	1.11	67.0
Saline, 4–7 hrs.	1 ml./kilo	1.0035	1.19	40.0
Acetylcholine	2			85.2
Acetylcholine	40	1.0165		314.5
Mecholyl	5	0.9851		
Mecholyl	7.5	1.0008	1.55	193.1
Mecholyl	10.0	0.9905	1.60	475.7
Carbachol	0.01	0.9968	1.05	21.3
Carbachol	0.05	0.9938		93.7
Carbachol	0.10	1.0026		369.2
Urecholine	0.5	1.0162	1.89	448.6
Furmethide	0.1	0.9953	1.29	106.0
Furmethide	0.3	0.9956		172.5
Physostigmine	0.01	0.9959		35.5
Physostigmine	0.5	0.9955		71.0
Physostigmine	0.75	0.9987		136.3
Prostigmine	0.2	0.9973	1.09	103.0
Pilocarpine	0.05	0.9945	1.13	57.5
Pilocarpine	0.25	0.9985		100.1
Pilocarpine	0.5	0.9969	1.69	200.9
Pilocarpine	1.0	1.0041	1.82	520.4
Arecoline	1.0	0.9961	1.80	204.5

administration. This was done upon the R.T.F. of most of the cats, changes in the volume output of which have been listed in table 1. The results were avaraged and these means are given in table 2.

Boyd et al. (11) have previously reported upon many of the normal physical and chemical properties of R.T.F., and the mean specific gravity of cat R.T.F. was shown to be 1.014. A somewhat lower mean was obtained in the present investigation: 0.9980 for the first 3 hours of collection of R.T.F. and 1.0035 for the 4th to 7th hours. The specific gravity of R.T.F., during the four hours after giving the drugs, was not markedly affected by injection of various doses of

parasympathomimetic drugs. The grand average of all means after giving parasympathomimetic drugs was 0.9985, in contrast to the mean after giving the control saline injection of 1.0035. While the difference is not striking, the mean specific gravities of R.T.F. after giving parasympathomimetic drugs were almost invariably lower than the specific gravity of the control R.T.F. It seems reasonable to conclude that parasympathomimetic drugs have little effect upon the specific gravity of R.T.F. but that what mean difference there is, is on the side of lowering of the specific gravity.

The relative viscosity of R.T.F. In a similar manner, the relative viscosity of R.T.F. collected 3 hours before and 4 hours after administration of the drugs, was determined using Ostwald viscosity pipettes. The mean results are recorded in table 2. The mean relative viscosity increased slightly in the control animals, from 1.11 during the first 3 hours to 1.19 from the 4th to 7th hours. Unfortunately it was not possible to estimate the relative viscosity in all experiments, but the results obtained definitely indicate that parasympathomimetic drugs, especially in the higher doses, augment the relative viscosity of R.T.F. The grand mean of all relative viscosities of R.T.F. after giving parasympathomimetic drugs was 1.49 in contrast to 1.19 in the control group and 70 per cent of the values were above that of the control mean. It may be concluded, therefore, that parasympathomimetic drugs, especially in higher doses, increase the relative viscosity of R.T.F.

The chloride content of R.T.F. Of the three physical and chemical properties of R.T.F. which we were able to study, parasympathomimetic drugs produced the greatest change in the chloride content of R.T.F. (table 2). In the control cats, the chloride content, estimated by Van Slyke's method (12), averaged 67 mgm. per 100 ml. of R.T.F. in the collection of the first three hours, which compares favourably with previously published reports (11). During the next four hours in the control cats, the chloride content fell to 40 mgm. per 100 ml. of R.T.F. In marked contrast, the chloride content of R.T.F. of all cats receiving parasympathomimetic drugs, rose and rose markedly as the doses of the drugs were increased. This occurred without exception in all instances. The grand mean of the individual averages for chloride content after giving parasympathomimetic drugs was 195 mgm. per 100 ml. of R.T.F. in contrast to 40.0 in the controls. It may be concluded that parasympathomimetic drugs produce an increase, and a marked increase as the dose is raised, in the chloride content of R.T.F. of cats.

SUMMARY

Approximately 400 animals, including albino rats, guinea-pigs, rabbits, cats and dogs, were arranged for the collection of respiratory tract fluid (R.T.F.). A range of doses of eleven parasympathomimetic drugs (acetylcholine, mecholyl, carbachol, urecholine, furmethide, physostigmine, prostigmine, the dimethylurethane of m-isopropyl-p-dimethyl-amino-phenol methiodide, the dimethylurethane of p-dimethylamino thymol methiodide, pilocarpine and arecoline) was administered subcutaneously.

Practically all the drugs increased the volume output of R.T.F., some to over 1,000 per cent.

Atropine sulphate eliminated this effect when produced by pilocarpine, selected as an example of this group.

Section of the cervical vagus nerve had no effect upon the action of pilocarpine in cats.

Pilocarpine was as effective in decerebrate as in urethanized cats.

There was some evidence that pilocarpine is less effective in the winter and spring than in the summer months.

The mean specific gravity of cat R.T.F. was slightly lowered by parasympathomimetic drugs.

The relative viscosity of cat R.T.F. was increased by parasympathomimetic drugs.

The chloride content of R.T.F. was markedly increased by parasympathomimetic drugs.

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PYRUVIC ACID ANTAGONISM TO BARBITURATE DEPRESSION

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The mechanism of the depressant action of barbiturates on the central nervous system is not clearly understood. However, evidence is accumulating to suggest some relationship between the action of this group of drugs and altered carbohydrate metabolism. Such a relationship might be expected, since Himwich and Nahum (1932) found the respiratory quotient of the dog brain to be about 1.00, indicating an extremely large proportion of carbohydrates (as compared to other metabolites) being metabolized by the brain. Studies of brain metabolism have revealed that the addition of pyruvate to brain tissue partially depleted of its autoxidizable material restores the rate of oxidation to a level equal or higher than that reached by adding glucose (Quastel and Wheatley, 1932). These writers in later work (1933) suggested that a characteristic feature of narcotic activity (including allylisopropylbarbituric acid and phenylethylbarbituric acid) is an inhibitory effect on the oxidations of glucose, sodium lactate and sodium pyruvate and little or none at all of that of sodium succinate. The literature however is somewhat confusing on a possible antagonism of barbiturate depression by succinate (Soskin and Taubenhaus, 1943; Lardy, Hansen, and Phillips, 1944; Beyer and Latren, 1944; Pinschmidt, Ramsey and Haag, 1945; Corson, Koppanyi and Vivino, 1945; DeBoer, 1946). Quastel (1936) suggested that narcosis itself is linked with the power of the narcotics to inhibit glucose or lactic acid oxidations at the nervous centers at which the narcotics are adsorbed, and that the narcotics do not interfere with the access or activation of oxygen by brain tissue. In an extensive review and discussion of the comparative biochemistry of carbohydrate metabolism, Barron (1943) was of the opinion that pyruvate is the most important intermediary product in carbohydrate metabolism, as it is the end product of the first anaerobic phase of fermentation and the first step of carbohydrate oxidation and synthesis. Dorfman (1943) in a detailed discussion of the pathways of glycolysis believes that pyruvic acid may be reduced to lactic acid, decarboxylated or oxidized by a great variety of reactions.

Experimental. The present study has been made in an attempt to determine whether pyruvic acid plays some rôle in the anesthetic effect of pentobarbital on rabbits. As it has been shown that hyperglycemia increases the pyruvate content of the blood (Bueding, Fazekas, Herlich, and Himwich, 1942, Bueding, Stein and Wortis, 1941; and Bueding and Goldfarb, 1943) and that injections of epinephrine into animals will raise both the blood sugar level and the pyruvic acid level (Schmidt, 1942), eight young rabbits were injected with a heavy anesthetic dose of sodium pentobarbital (40-mgm./kgm. I.V.) followed by an intramuscular injection of epinephrine hydrochloride (0.05 cc of 1–2,000/kgm.) to determine the effect epinephrine might have on the depression produced by the pentobarbital. The sleeping time is defined as the interval between the injection of the rabbit with pentobarbital and the time that the rabbit would no longer remain on its back. The anesthetized rab-

bits were placed on their backs and every five minutes rolled over and back again as a mild stimulus to assure a better end point. The control group of eight rabbits received the same treatment, omitting the epinephrine.

TABLE 1

a. Sleeping time of rabbits injected with pentobarbital sodium.*

RABBIT NUMBER	WT.	BEFORE PENTOBARBITAL BLOOD SUGAR	ONE HOUR AFTER INJECTION BLOOD SUGAR	ON AWAKENING BLOOD SUGAR	SLEEPING TIME
	kgm.	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*minutes*
1	1.64	106	104	98	139
2	1.49	100	97	95	128
3	1.89	97	100	100	90
4	1.89	97	98	91	93
5	1.70	103	100	100	70
6	1.41	95	89	79	115
7	1.10	97	96	84	118
8	1.70	103	98	85	110
Average	1.60	100	98	92	108

b. Sleeping time of rabbits injected with pentobarbital sodium* and epinephrine.†

9	2.09	101	193	230	78
10	2.27	89	133	133	60
11	2.13	105	196	196	60
12	2.13	110	161	169	79
13	1.63	114	140	139	87
14	1.68	105		184	49
15	1.63	104	159	159	60
16	1.68	105	149	150	75
Average	1.91	104	141	172	68

c. Sleeping time of rabbits injected with pentobarbital sodium* and insulin.‡

17	1.59	104		61	44
18	1.52	91	78	78	62
19	1.80	98	75	75	62
20	1.59	104		53	56
21	1.63	106		52	56
22	1.86	106	41	41	61
23	1.47	97	42	42	61
24	1.54	100		71	54
Average	1.63	101	59	59	57

* 40 mgm. per kgm.

† 0.05 cc of 1–2,000 epinephrine hydrochloride per kgm.

‡ 1 unit per kgm.

Table 1 shows an average sleeping time of 108 minutes, for the control animals and an average blood sugar level of approximately 100 mgm. per cent both before and after the injection of pentobarbital. However, in table 2 the average blood

sugar level rose from 104 mgm. per cent before injection of the pentobarbital and epinephrine to 164 mgm. per cent one hour after injection of those drugs, and to 172 mgm. per cent at the time of awakening. The average sleeping time was only 68 minutes as compared to 108 minutes for the control rabbits.

From the data in table 1 it is not evident whether the shortened sleeping time due to epinephrine results directly from the increased blood sugar or a greater supply of pyruvate. Therefore, to distinguish between these possibilities, insulin was injected into rabbits, for insulin has been shown to increase the metabolism of pyruvic acid injected intravenously into rabbits (Delrue and Dekeyeser, 1940; although Elliot, Rivers, Elliott and Platt in 1941 found no change in man) and to decrease the level of pyruvate in the blood (Nylin, Euler and Hogberg, 1939; Euler, and Hogberg, 1940). Eight rabbits were injected with pentobarbital sodium (40 mgm./kgm.) followed immediately by an intramuscular injection of insulin (1 unit/kgm. I.M.). Table 1 shows that these animals slept an average of only 57 minutes and had an average blood sugar level of 59 mgm. per cent at the time of waking. By comparing the sleeping time and the high blood sugar level of the anesthetized rabbits receiving epinephrine with the still shorter sleeping time, but very low blood sugar level in those animals receiving insulin, it is obvious that the blood sugar level alone is not the determining factor as regards sleeping time under barbiturate anesthesia.

As epinephrine and insulin each will increase tissue oxidation (Ahlgren 1924; Euler, 1930), and epinephrine will increase the pyruvate level of the blood and as insulin has been found to increase pyruvate metabolism, thus lowering the pyruvate content of the blood, and both have been found by the writer to shorten the sleeping time in rabbits anesthetized with pentobarbital sodium, it seemed logical that some sort of utilization of pyruvic acid might be the step in carbohydrate metabolism being depressed by the barbiturate compound. Therefore, if the supply of pyruvic acid in the blood were increased by injection, the action of the pyruvate might be increased and the intensity or duration (or both) of depression due to pentobarbital correspondingly decreased.

Therefore, in an exploratory experiment eight rabbits were injected intravenously with pentobarbital sodium (40 mgm./kgm.) and then immediately with pyruvic acid (5 mgm./kgm. in 0.85 per cent saline solution). This group of animals reacted in a manner such that the endpoint for the sleeping time was very difficult to obtain. The time elapsing between injection and refusal to stay on the back was much shorter, but there followed a long period of stupor during which no spontaneous movement occurred. When the sleeping time was added to this period of stupor the total time thus obtained (referred to hereafter as "total depression time") was practially as long as the sleeping time of the controls. This experiment was repeated on a group of 16 young rabbits, and a group of 16 adult rabbits, specific attention being given to sleeping time and total depression time. The results are shown in table 2. The sleeping time in the control animals averaged 99 minutes with only 4 additional minutes elapsing before spontaneous movements occurred, whereas in the group of young animals receiving the pyruvic acid the sleeping time averaged only 73 minutes with 29 minutes more required before spontaneous movements were noted. The sleeping time of the

adult control animals averaged 97 minutes while that of the experimental group was only 54 minutes. Again the very short additional time of 5 minutes for the control animals as compared to 42 for those receiving pyruvic acid was observed before spontaneous movements occurred after the sleeping time had ended. The total depression time in the young animals was 103 minutes compared to 102 minutes for the experimental group. Similarly, 103 minutes for the total depression time of the adult control group and 96 minutes for the experimental animals were observed.

TABLE 2

Effects of pyruvic acid injection on sleeping time and total depression time in rabbits anesthetized with pentobarbital sodium

RABBIT NUMBER	WT.	SLEEPING TIME	TOTAL DEPRESSION TIME	RABBIT NUMBER	WT.	SLEEPING TIME	TOTAL DEPRESSION TIME
a. Young rabbits receiving pentobarbital sodium				Young rabbits receiving pentobarbital sodium and pyruvic acid			
	kgm.	*minutes*	*minutes*		*kgm.*	*minutes*	*minutes*
25	3.00	131	133	33	3.00	66	88
26	2.36	132	136	34	2.36	77	99
27	2.50	102	111	35	2.45	71	97
28	2.60	79	85	36	2.36	73	106
29	3.09	100	100	37	3.10	78	109
30	2.50	86	87	38	2.45	80	112
31	2.89	61	65	39	2.71	75	99
32	2.90	108	108	40	2.25	67	107
Average.....	2.73	99	103	Average......	2.57	73	102
b. Adult rabbits receiving pentobarbital sodium.				Adult rabbits receiving pentobarbital sodium and pyruvic acid.			
41	4.10	100	105	49	3.20	38	98
42	4.10	135	146	50	3.80	67	122
43	4.00	145	149	51	4.05	41	102
44	3.00	90	93	52	3.15	66	88
45	4.05	70	75	53	3.00	49	84
46	3.20	71	76	54	4.00	64	114
47	3.15	75	76	55	4.10	42	80
48	3.80	97	103	56	4.10	67	84
Average.....	3.68	97	103	Average......	3.67	54	96

As the same dose of pyruvic acid injected into each of 6 rabbits having no pentobarbital produced no apparent depressing effect, the stupor observed in the above experimental group receiving pentobarbital could not be due to simple hyperpyruvaemia.

DISCUSSION. The data presented indicate an inverse relationship between the amount of pyruvic acid available in the blood and the degree of depression produced in rabbits by pentobarbital sodium. Although the total depression time was not altered materially by the pyruvic acid injection, the shortened sleep-

ing time as judged from the rabbit's failure to remain on its back after having had its position changed, coupled with various responses characteristic of light stages of anesthesia by the experimental animals, and not shown by the control rabbits during the sleeping time, indicated an antagonistic effect between the pyruvic acid and pentobarbital sodium. Three possible explanations for this inhibition of depression present themselves, namely, the higher pyruvic acid level may have increased the rate of excretion of the barbiturate, or the rate of destruction of the pentobarbital sodium, or the action of pyruvic acid or its breakdown products. As the total depression time was relatively unchanged the rate of destruction or excretion of the barbiturate probably was not altered to any great extent, for it is known that smaller doses not only depress an animal to a lesser degree, but also persist for a shorter time. Therefore, it would appear that the decreased depth of depression of the animals by the barbiturate when followed by the injection of pyruvic acid results from an antagonism of the pyruvate against the action of the pentobarbital.

SUMMARY

Data on 64 rabbits anesthetized with pentobarbital sodium (40 mgm./kgm. intravenously) indicate that:

1. Epinephrine increases the blood sugar level and shortens the sleeping time.
2. Insulin decreases the blood sugar level and shortens the sleeping time.
3. Intravenous injection of pyruvic acid decreases the depth of depression, and shortens the sleeping time (as defined in this study), but does not alter materially total depression time in rabbits.

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THE EFFECTS OF SENECIONINE ON THE MONKEY

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Senecionine is an alkaloid occurring in several species of *Senecio*: *S. vulgaris*, *S. viscosus*, *S. squalidus*, *S. integerrimus*, *S. aureus*, and *S. pseudo-arnica* (1). Harris, Anderson, and Chen (2) found that intravenous injection of a single dose of senecionine in mice was followed by necrosis of the liver cells, sinusoidal congestion, and hemorrhage into the hepatic cell cords. In addition, hydrothorax, ascites, and pulmonary edema occurred in some mice. It was also observed that senecionine when injected intravenously into rats prolonged the plasma prothrombin time (3).

The present study was undertaken to ascertain what effect senecionine would have on prothrombin time, the liver, and other organs, of a mammal closer to man in the evolutionary scale, namely, the monkey (*Macaca rhesus*).

The specimen of senecionine was generously supplied by Dr. Richard H. F. Manske, formerly of the Division of Chemistry, National Research Council, Ottawa, Canada. It was isolated from *S. vulgaris* (4) and *S. integerrimus*. Solutions were made by dissolving the weighed quantities in equimolecular amounts of hydrochloric acid.

METHODS. Four monkeys of various ages weighing between 3.3 and 8.8 kilograms were used in this study. Control plasma prothrombin times were taken before the intravenous administration of senecionine, and at intervals thereafter until the animal succumbed to the toxic effects of the drug. Plasma prothrombin determinations were made as described previously (3). A 2 per cent solution of senecionine was slowly administered into a vein of one of the limbs. The initial dose varied from 10 to 30 mg. per kg. It was repeated—usually daily until the animal became apparently ill. To avoid postmortem changes, necropsy was made as soon as possible after, or on the point of, death, and specimens were taken from various organs for histopathologic study.

Three additional rhesus monkeys were sacrificed, and their organs were removed for microscopic examination. They served as controls. This enabled us to compare carefully the normal organs and those under the influence of senecionine.

RESULTS. 1. *Effects on prothrombin activity.* The effects of senecionine on the prothrombin activity of dilute (12.5 per cent) plasma in 4 monkeys are summarized in table 1. In monkeys numbered 1, 2, and 4, a progressive prolongation of prothrombin time was observed. In monkey numbered 3, there was a slight prolongation of the prothrombin time during the first day after the initial dose of senecionine, but a return to normal occurred thereafter even though the animal succumbed on the ninth day, after having received 4 injections of senecionine totaling 75 mg. per kg. of body weight.

Accompanying the depression of plasma prothrombin there was a progressive increase in serum bilirubin. This rise in blood bilirubin was also obtained in the monkey whose plasma prothrombin was not much affected by senecionine. Monkeys numbered 1 and 2, after administration of the drug, had clonic con-

vulsions which lasted several minutes. All the animals were depressed and prostrated for long periods before death.

TABLE 1

Effects of senecionine on prothrombin activity

MONKEY NUMBER	SURVIVAL TIME	TOTAL DOSE, INTRAVENOUSLY	PROTHROMBIN TIME IN SECONDS ON 12.5 PER CENT PLASMA										
			Control	Time in hours after first dose									
				1	3	5	24	25	27	29	48	53	At death
	days	*mg. per kg.*											
1	3	50	50	50	44	47	54	56	60	81	81	122	161
2	3	30	65	76	84	97	107		127	153	200	261	366
3	9	75	68	72	78	84	90	80	76	80	65	67	
4	3	60	41				76			160	330	397	397

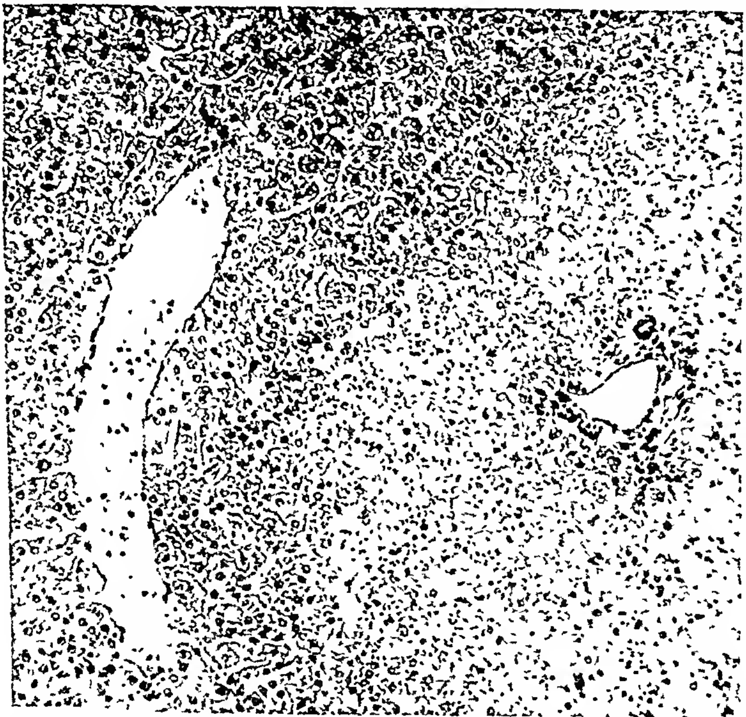

FIG. 1. LIVER NECROSIS PRODUCED BY SENECIONINE. ×120

Monkey numbered 4, female, weighing 3.3 kg., was injected intravenously 2 doses of senecionine, 30 mg. per kg. on 2 consecutive days. It died on the third day. There is a broad band of periportal necrosis at the right, but a few viable liver cells persist immediately about the portal space.

2. *Necropsy findings.* The lesions in monkeys numbered 1, 2, and 4 were similar, and for that reason one description will suffice for all. The tissues were

slightly icteric. The liver was friable and diffusely yellow. In some regions, the lobules were indistinguishable, but in the greater portion of the tissue the lobules were rendered very conspicuous by a dull, yellow, opaque network which contrasted strongly with the more translucent brown tissue in the interstices. In many places a delicate red network was seen in the opaque tissue. In monkey numbered 2, petechiae were seen in the peritoneal surfaces, epicardium, and left ventricular endocardium. There were also a few small erosions of the mucosa

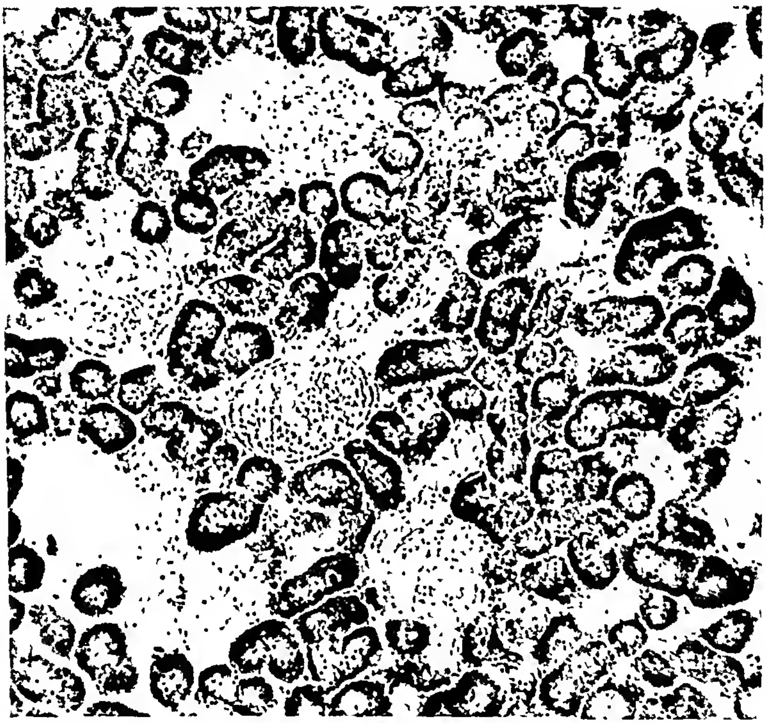

FIG. 2. FATTY DEGENERATION OF THE KIDNEY. ×120

Monkey numbered 2, female, weighing 8.5 kg, was injected intravenously a single dose of senecionine, 30 mg. per kg. It died on the third day. The section of the kidney was stained with scarlet red alone. Cells of the proximal convoluted tubules contain many small fat vacuoles. The glomeruli contain very little fat. Some fat-free distal tubules are included.

of the fundus of the stomach. A few petechiae were also observed in the left ventricular endocardium of monkey numbered 4. The other viscera of these 3 animals were not grossly abnormal.

Microscopic examination revealed significant changes in the liver and the kidneys only. There was extensive midzonal and periportal necrosis, usually with only a few viable liver cells persisting about the portal spaces and broad bands of viable cells about the central vein. An example is shown in figure 1.

In some places, necrosis was more extensive, and only a few viable cells surrounded the central veins. Occasionally, necrosis involved all liver cells of several contiguous lobules. In places, hemorrhage had occurred into the cords of necrotic cells, but in no section was it extensive. Much fat was present in the necrotic cells as well as in the viable ones. Leukocytic infiltration of necrotic cells was rarely encountered, and, when present, it was never found to be heavy.

The cells of the renal convoluted tubules of all 3 monkeys contained fat droplets, the proximal tubules showing considerably more and larger droplets than the distal tubules. This condition was most striking in animal numbered 2, and was least pronounced in animal numbered 4. The fat deposition is very obvious in figure 2. The presence of fat droplets was confirmed by scarlet red stain. The kidneys of 3 untreated monkeys, studied at the same time, were devoid of fat droplets.

The liver of monkey numbered 3 showed neither necrosis nor fatty metamorphosis. In fact, it appeared entirely normal. This is in harmony with the observation that the prothrombin time of the same animal became normal. The cells of the renal proximal convoluted tubules, like those of the other 3 monkeys, contained many fat globules; and the cells of the distal convoluted tubules, smaller numbers of minute fat globules. There was hemorrhage in the center of the left adrenal gland of this monkey. Microscopically, it was seen to have occurred at the cortico-medullary junction; and at the periphery, small numbers of fibroblasts were easily made out. This lesion of the adrenal gland, however, could have been accidental.

A point of some interest, with reference to the hepatic injury, is that in rats and mice necrosis occurred primarily in the central part of the lobule, whereas in the monkey the cells in the center of the lobule were the least susceptible to the action of senecionine. The reason for this difference is problematic, but herein we have another example of the well-known variation in susceptibility to injurious agents among animals of different species.

SUMMARY

Intravenous administration of senecionine to 4 rhesus monkeys led to the development of extensive necrosis of the liver of 3 of the animals, and to fatty degeneration of the kidneys of all 4. Associated with the hepatic necrosis were a steady increase of prothrombin time and a progressive elevation of the serum bilirubin.

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COMPETITIVE INHIBITION OF PROCAINE CONVULSIONS IN GUINEA PIGS

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The marked reduction of the chemotherapeutic effect of sulfonamides by the presence of pus and cell detritus has been explained by their content of p-aminobenzoic acid (PABA). This substance supposedly competes with the sulfonamides for the same receptors in the metabolic processes of the bacteria, due to the chemical similarity of the structues of PABA and the sulfonamides (1); or interferes on the basis of this similarity with certain catalytic processes (2 and 3). It was soon discovered that other drugs containing the PABA structure also inhibit the therapeutic action of sulfonamides. The most important examples are local anesthetics which are derived from PABA, particularly procaine and its congeners.

There is good experimental evidence (4 and 5) that procaine, being an ester of PABA with diethylaminoethanol, is easily split in the body into these components. This is probably the first step in the metabolic disintegration of this compound. In a study of the mechanism of the convulsive action of local anesthetics, it seemed possible that the presence of large amounts of PABA in the body might perhaps antagonize these central effects of procaine in a similar manner as in the case of PABA and sulfonamides in bacteria.

Experimental. In agreement with Beutner (6), we found the guinea pig a very suitable animal for the study of procaine-induced convulsions. No tolerance formation to the convulsive action developed in our experiments, where the animals were allowed at least four days' rest between tests. Animals of both sexes, usually weighing between 300 and 500 gm., were used. They were kept on a diet of rabbit pellets with ample fresh greens added. The importance of a satisfactory Vitamin C supply will be reported in another publication (7). Injections with local anesthetics were made intramuscularly into the hind legs and care was taken not to puncture blood vessels. A large percentage of the animals were injected first with the local anesthetics alone to determine their sensitivity before being used in the following experiments. Frequently their convulsive reactions to the local anesthetic was rechecked after they had served in several tests.

The bulk of the data obtained in these experiments is presented in table 1 The intramuscular injection of 100 mg./kg. = 0.365 mM procaine hydrochloride, corresponding to 0.425 mM/kg. of the base as a 6% solution, produced convulsions in 83% of the animals. This result is the average of all groups of animals usually consisting of 10–12 guinea pigs, and is in agreement with our previous experiences with this species (8). The convulsions began 5 to 10 minutes after injection and the tonic-clonic seizures of a moderate to severe degree lasted 5 to 25 minutes and were non-fatal.

The toxicity of PABA is quite low (9). The fatal dose in guinea pigs was not determined but it was found that intraperitoneal or intravenous injections of 800

mg./kg. of a 10% PABA solution, neutralized to a pH of 6–7.5 with NaOH, produced no noticeable symptoms in this species. In most experiments, we injected the drug intraperitoneally 30 to 35 minutes before the intramuscular in-

TABLE I

*Inhibition of procaine convulsions in guinea pigs by p-amino benzoic acid and some of its derivatives**

NO.	DRUG TESTED (MOL. WEIGHT)			PROCAINE HCl		NO. CONV. / NO. INJ.	% CONV.	χ^2 ABOVE 3.84?	REMARKS
		mg./kg.	mM/kg.	mg./kg.	mM/kg				
1				100	0.364	35/42	83		tonic clonic convulsions
				125	0.456	25/25	100		tonic clonic convulsions
2	p-Amino Benzoic Acid $C_6H_4(NH_2)$-COOH (137)	400	2.9	100	0.364	28/50	56	Yes	mild convulsions
		600	4.4	100	0.364	4/30	13	Yes	mild convulsions
		800	5.8	125	0.456	6/16	37	Yes	p.a.b. given i.v. 5 min. before proc.
3	o-Amino Benzoic Acid (137)	400	2.9	100	0.364	9/13	69	No	
		600	4.4	100	0.364	25/30	83	No	
4	p-Hydroxy Benzoic Acid $C_6H_4(OH)$-COOH (138)	470	3.4	100	0 364	9/19	48	Yes	predominantly mild convulsions
		622	4.5	100	0 364	15/27	55	Yes	predominantly mild convulsions
5	o-Hydroxy Benzoic Acid (138)	470	3.4	100	0 364	12/12	100	No	1 fatal
		622	4 5	100	0.364	10/12	83	No	2 fatal
6	Benzoic Acid C_6H_5-COOH (122)	600	4.9	100	0.364	5/15	33	Yes	predominantly mild convulsions
7	Sulfathiazole (255)	640	2.5	100	0.364	9/12	75	No	ST given 45 min. prior to Procaine
8	Sulfanilamide (172)	1000	5.8	100	0.364	11/11	100	No	SA given in 2 doses of 500 mg./kg. 1–2 hrs. prior to proc.

* Injected intraperitoneally 30–35 minutes prior to intramuscular injection of Procaine HCl except the last dose of (2)—see under "remarks."

Drugs Nos. 2–6 were given as Na salts of a pH of 6–7.5.

Drugs Nos. 7–8 were dissolved with NaOH.

jection of the convulsant; intravenous administration of PABA immediately before the intramuscular injection of procaine was made in only one group (table 1).

With this technique, no significant effect upon procaine convulsions was produced by premedication with 200 mg./kg. PABA as sodium salt; however, with 400 mg./kg. = 2.9 mM/kg., only 56% had convulsions. The onset of the seizures in this group was frequently delayed for fifteen minutes and their severity was greatly lessened.[1] With a dose of 600 mg./kg. PABA, a further reduction of the convulsions to 13% was obtained. If 800 mg./kg. PABA were given intravenously five minutes before the intramuscular injection of 125 mg./kg. procaine, only 37% of the animals experienced convulsions against 100% in untreated animals. A statistical analysis of the significance of the data was made by the χ^2 test, using 2 x 2 tables, comparing the groups pretreated with the different drug, with the group injected with procaine alone. The Yates correction was employed where desirable. A χ^2 value of above 3.84 was taken as indicating a significant difference.

In order to determine the degree of specificity of the protective effect produced by PABA, we next tested several related compounds as listed in table 1. Ortho-aminobenzoic acid proved to be ineffective if used at the same dose levels as the para form. Comparable amounts of either benzoic or p-hydroxybenzoic acid exerted some protective action, but not o-hydroxybenzoic acid.

Two members of the sulfonamide series failed to show protective action with the use of either smaller (sulfathiazole) or larger (sulfanilamide) mole equivalents than PABA itself.

After it was thus shown that premedication with a large amount of PABA inhibited the convulsant action of procaine, a local anesthetic derived from this substance, the question arose as to what effect, if any, would be produced if the other part of the molecule, namely the aminoalcohol, were used in place of the PABA (table 2).

The procedure was the same as that described. All amino alcohols were neutralized with hydrochloric acid and injected intraperitoneally 30 minutes before the local anesthetic. Injections of 400 mg./kg. or 3.42 mM/kg. of diethylaminoethanol (DEAE) produced no symptoms. However, if followed by 100 mg./kg. procaine, the incidence of convulsions dropped to 8.8% (table 2, No. 1). Reduction of the alcohol to 200 mg./kg. resulted in the loss of a protective effect.

The fact that premedication with DEAE exerted a marked protective action invited the study of other amino alcohols having a longer or shorter alcoholic group or with the substitution of the alkyl radicals. The extent of this work was limited by the availability of these compounds and by toxic effects produced by higher homologues. Already, Richardson (10) had observed an increasingly toxic action of alcohols with lengthening of the chain. This work was later confirmed by other investigators as Dold (11) and Macht (12). Hauschild (13) has studied some pharmacological actions of a selected number of alkyl and alcanol amines. The amino alcohols did not produce toxic symptoms in the experiments listed on table 2 except where otherwise indicated.

[1] In preliminary experiments (Richards, R. K., J. Biol. Chem., 159: 241, 1945) only 15% of the animals pretreated with 400 mg./kg. PABA had convulsions. These data are not incorporated in the experiments listed on table 1.

Since 3.4 mM/kg. DEAE were found protective, this dose was also used as the standard amount for the other compounds as shown in table 2. As can be seen, increase of the chain by one CH_2 did not destory the protective action. In fact, β-diethylaminopropanol (No. 2) is as effective as DEAE itself while the γ-ana-

TABLE 2

Effect of various N-substituted amino alcohols upon procaine convulsions in guinea pigs

COMPOUND TESTED (MOLECULAR WT.)			NUMBER CONV./NUMBER USED	% CONV	χ^2 ABOVE 3.84	REMARKS
	mg/kg.	mM/kg	%			
1. $(C_2H_5)_2NCH_2CH_2OH$ (117)	200	1.71	7/10	70.0	No	Mild convulsions
	400	3.42	3/34	8.8	Yes	Mild convulsions
2. $(C_2H_5)_2NCH(CH_3)CH_2OH$ (131)	435	3.4	1/14	7.2	Yes	Mild convulsions
3. $(C_2H_5)_2N(CH_2)_2CH_2$-OH (131)	435	3.4	5/25	20.0	Yes	Mild convulsions
4. $(C_2H_5)_2N(CH_2)_3CH_2$-OH (141)	494	3.4	11/15	73.0	No	
5. $(CH_3)_2NCH_2CH_2OH$ (89)	300	3.4	9/10	90.0	No	
6. $C_2H_5NHCH_2CH_2OH$ (89)	300	3.9	7/10	70.0	No	
7. $C_4H_9NHCH_2CH_2OH$ (117)	398	3.4	5/20	25.0	Yes	One animal died
8. $(C_3H_7)_2NCH_2CH_2OH$ (145)	225	1.5	8/24	33.0	Yes	Two animals died
9. $C_2H_5N(CH_2CH_2OH)_2$ (133)	453	3.4	8/20	40.0	Yes	

The compounds were injected intraperitoneally as hydrochlorides 30 minutes prior to intramuscular injections of procaine hydrochloride.

logue (No. 3) is less so. The next homologue diethylaminobutanol (No. 4) was no longer protective.

Substitution of both ethyl groups by methyl (No. 5) lead to a complete loss of effectiveness. The monoethylaminoethanol (No. 6) was likewise inactive. However, the monobutyl derivative (No. 7), an isomer of DEAE, proved definitely protective. This compound produced some toxic effects in the animals

With this technique, no significant effect upon procaine convulsions was produced by premedication with 200 mg./kg. PABA as sodium salt; however, with 400 mg./kg. = 2.9 mM/kg., only 56% had convulsions. The onset of the seizures in this group was frequently delayed for fifteen minutes and their severity was greatly lessened.[1] With a dose of 600 mg./kg. PABA a further reduction of the convulsions to 18% was obtained. If 800 mg./kg. PABA were given intravenously five minutes before the intramuscular injection of 125 mg./kg. procaine, only 37% of the animals experienced convulsions against 100% in untreated animals. A statistical analysis of the significance of the data was made by the χ^2 test, using 2 x 2 tables, comparing the groups pretreated with the different drug, with the group injected with procaine alone. The Yates correction was employed where desirable. A χ^2 value of above 3.84 was taken as indicating a significant difference.

In order to determine the degree of specificity of the protective effect produced by PABA, we next tested several related compounds as listed in table 1. Orthoaminobenzoic acid proved to be ineffective if used at the same dose levels as the para form. Comparable amounts of either benzoic or p-hydroxybenzoic acid exerted some protective action, but not o-hydroxybenzoic acid.

Two members of the sulfonamide series failed to show protective action with the use of either smaller (sulfathiazole) or larger (sulfanilamide) mole equivalents than PABA itself.

After it was thus shown that premedication with a large amount of PABA inhibited the convulsant action of procaine, a local anesthetic derived from this substance, the question arose as to what effect, if any, would be produced if the other part of the molecule, namely the aminoalcohol, were used in place of the PABA (table 2).

The procedure was the same as that described. All amino alcohols were neutralized with hydrochloric acid and injected intraperitoneally 30 minutes before the local anesthetic. Injections of 400 mg./kg. or 3.42 mM/kg. of diethylaminoethanol (DEAE) produced no symptoms. However, if followed by 100 mg./kg. procaine, the incidence of convulsions dropped to 8.8% (table 2, No. 1). Reduction of the alcohol to 200 mg./kg. resulted in the loss of a protective effect.

The fact that premedication with DEAE exerted a marked protective action invited the study of other amino alcohols having a longer or shorter alcoholic group or with the substitution of the alkyl radicals. The extent of this work was limited by the availability of these compounds and by toxic effects produced by higher homologues. Already, Richardson (10) had observed an increasingly toxic action of alcohols with lengthening of the chain. This work was later confirmed by other investigators as Dold (11) and Macht (12). Hauschild (13) has studied some pharmacological actions of a selected number of alkyl and alcanol amines. The amino alcohols did not produce toxic symptoms in the experiments listed on table 2 except where otherwise indicated.

[1] In preliminary experiments (Richards, R. K., J. Biol. Chem., 159: 241, 1945) only 15% of the animals pretreated with 400 mg./kg. PABA had convulsions. These data are not incorporated in the experiments listed on table 1.

Since 3.4 mM/kg. DEAE were found protective, this dose was also used as the standard amount for the other compounds as shown in table 2. As can be seen, increase of the chain by one CH_2 did not destory the protective action. In fact, β-diethylaminopropanol (No. 2) is as effective as DEAE itself while the γ-ana-

TABLE 2

Effect of various N-substituted amino alcohols upon procaine convulsions in guinea pigs

COMPOUND TESTED (MOLECULAR WT.)			NUMBER CONV. / NUMBER USED	% CONV.	χ^2 ABOVE 3.84	REMARKS
	mg./kg.	mM/kg.	♀			
1. $(C_2H_5)_2NCH_2CH_2OH$ (117)	200	1.71	7/10	70.0	No	Mild convulsions
	400	3.42	3/34	8.8	Yes	Mild convulsions
2. $(C_2H_5)_2NCHCH_2OH$ (131) with CH_3 on CH	435	3 4	1/14	7.2	Yes	Mild convulsions
3. $(C_2H_5)_2N(CH_2)_2CH_2$-OH (131)	435	3.4	5/25	20.0	Yes	Mild convulsions
4. $(C_2H_5)_2N(CH_2)_3CH_2$-OH (141)	494	3.4	11/15	73.0	No	
5. $(CH_3)_2NCH_2CH_2OH$ (89)	300	3.4	9/10	90 0	No	
6 $C_2H_5NHCH_2CH_2OH$ (89)	300	3.9	7/10	70 0	No	
7. $C_4H_9NHCH_2CH_2OH$ (117)	398	3 4	5/20	25 0	Yes	One animal died
8. $(C_3H_7)_2NCH_2CH_2OH$ (145)	225	1.5	8/24	33 0	Yes	Two animals died
9. C_4H_9N with two CH_2CH_2OH groups (133)	453	3.4	8/20	40.0	Yes	

The compounds were injected intraperitoneally as hydrochlorides 30 minutes prior to intramuscular injections of procaine hydrochloride

logue (No. 3) is less so. The next homologue diethylaminobutanol (No. 4) was no longer protective.

Substitution of both ethyl groups by methyl (No. 5) lead to a complete loss of effectiveness. The monoethylaminoethanol (No. 6) was likewise inactive. However, the monobutyl derivative (No. 7), an isomer of DEAE, proved definitely protective. This compound produced some toxic effects in the animals

as evidenced by some depression and one died after the procaine was administered. Propylaminoethanol (No. 8) produced marked excitement and frequently death if 3.4 mM/kg. were injected. Such symptoms were absent or mild with 1.5 mM/kg. and this dose reduced the procaine convulsions to 33%. However, a definite depression was noticed in this group and two animals died. Of interest is the presence of a moderate protective effect with compound No. 9 containing two ethanol and one ethyl group on the tertiary N atom.

In order to determine the duration of the protective effect, two groups of 15 guinea pigs each were given 400 or 600 mg./kg. PABA respectively one hour before the adminstration of 100 mg./kg. procaine HCl. Under these circumstances, PABA was no longer effective since 80% of the first and 67% of the second group had convulsions. The occurrence of 40% convulsions in the identical experiments with 400 mg./kg. DEAE indicated that a diminished but significant degree of protection was still present one hour after the injection of the drug.

Since it became evident from the above reported experiments that either of the two mother substances of procaine alone would effectively prevent the convulsions caused by this local anesthetic, it remained to be investigated what effect would follow the simultaneous administration of PABA and DEAE. Vincent and Detrie (14) had found that the salt of PABA and DEAE is several times less toxic than the ester, namely procaine. Our preliminary experiments revealed that in several instances guinea pigs died if a mixture of the two drugs consisting of 200 mg./kg. PABA as the sodium salt and 172 mg./kg. DEAE as the hydrochloride, was injected. Death was due to general depression and delayed for several hours; no convulsions were noted. Further experiments indicated the increase of the toxicity of such mixture with prolonged standing, while freshly prepared combinations were sometimes tolerated without symptoms or fatalities. If the two compounds were injected simultaneously but separately, no toxic symptoms followed.

For the first series of experiments, we injected the guinea pigs with 4 mM of PABA plus 4 mM of DEAE for each mM of procaine. This relation represents equivalents of four hydrolyzed molecules of procaine for one intact molecule. Since we had found that 100 mg./kg. or 0.364 mM of procaine hydrochloride produced convulsions in 83% of the animals, the guinea pigs were injected injected intraperitoneally with 1.46 mM/kg. of each of the split products, amounting to 200 mg./kg. PABA and 172 mg./kg. DEAE. This was followed 30 minutes later by procaine given intramuscularly. Only 1 of 25 guinea pigs or 4% showed mild convulsions. Encouraged by this marked protection we reduced the doses of the PABA and DEAE to one-half, so that the split products of two molecules were used for each whole molecule of procaine. Under these conditions 8 of 25, or 32%, of the animals developed mild convulsions, indicating the presence of a considerable protective action by this procedure.

We have shown earlier in this paper that a rise of the dose of procaine hydrochloride by 25 mg. to 125 mg./kg. produced convulsions in all guinea pigs so treated. If this injection was preceded by the intraperitoneal administration of the equivalent of 4 molecules of each of the split products, namely

PABA and 215 mg./kg. DEAE, for each molecule of procaine, only 4 of 10 animals developed convulsions.

The effectiveness of simultaneous administration of the protective agents with procaine was also tried. The procaine in such a mixture is considerably more dilute than the 6% concentration in which it was used in previous experiments. Since it is known that the toxicity of such local anesthetics is often decreased by lowering the concentration (15), 2 series of experiments were carried out. The first consisted of 10 guinea pigs which were injected intramuscularly with a mixture consisting of 130 mg./kg. procaine hydrochloride as a 6% solution with 200 mg./kg. PABA and 127 mg./kg. DEAE, as 10% solutions. Six of these animals had convulsions. The second group of ten guinea pigs received the same dose of procaine hydrochloride diluted with saline to correspond in volume to that used in the first group. This resulted in 100% convulsions. We can conclude from these observations that simultaneous intramuscular administration of the inhibitor produced some protection but less so than by injection 30 minutes prior to the local anesthetics.

The following experiments were intended to investigate if compounds found effective in preventing the central convulsive action of local anesthetics would exert an antagonistic effect upon the typical local anesthetic action upon peripheral nerves.

a) Nerve muscle preparations of frogs were arranged in the usual manner and stimulated with submaximal single break shocks or short bursts of tetanizing currents. Parts of about 1 cm. length of the nerve were exposed to neutral solutions of 3 to 4% PABA or a mixture of 1.5 to 2.0% of both PABA and DEAE for about 10 minutes. Such preparations which showed no or little effect of these solutions were then exposed to identical solutions containing 1% procaine hydrochloride. Typical depression due to the local anesthetic occurred in all instances and in about the same time and to the same degree as with 1% procaine hydrochloride alone.

b) Intradermal wheals were placed in the back of guinea pigs using a 4% neutralized solution of either PABA or DEAE: after a few minutes had elapsed, a 1% solution of procaine hydrochloride was injected into these wheals. In other experiments, a mixture of the local anesthetic with either PABA or DEAE was used. In none of these tests did a noticeable reduction of the local anesthetic action occur.

Thus we were unable to demonstrate in these tests the presence of a peripheral antagonism of the typical local anesthetic effect of procaine by compounds found to interfere with their convulsive action.

Since PABA and DEAE had shown protective action against the convulsive effect of procaine, the questions arose if this anticonvulsive property was specific and to what degree. To exclude the possibility of a general anticonvulsive action of these two compounds, 400 mg./kg. PABA were injected intraperitoneally in guinea pigs, followed 30 minutes later by 75 mg./kg. metrazol intramuscularly, a dose which produced 85% convulsions in untreated animals. Typical metrazol convulsions occurred also in all guinea pigs injected with PABA prior to the met-

razol. Similar experiments in mice gave identical results. Like PABA, the administration of 400 mg./kg. DEAE failed to prevent the appearance of metrazol convulsions.

Diothanc (piperidinopropanedioldiphenylurethane) is a local anesthetic chemically unrelated to procaine. It caused moderate convulsions followed by marked depression in 7 of 10 guinea pigs (70%) when given intramuscularly in a dose of 150 mg./kg. = 0.29 mM/kg. In order to preserve the same molar relationships between PABA and Diothane as in the corresponding experiments with procaine, 2.3 mM/kg. or 320 mg./kg. PABA were used for pretreatment; however, 8 of 10 or 80% of the guinea pigs developed convulsions after the injection with Diothane. Increase of the PABA to 420 mg./kg. = 3.1 mM/kg. protected none of 6 animals so treated against convulsions.

These results support the view that PABA and DEAE are not general anticonvulsants and do not antagonize convulsions due to local anesthetics having a structure entirely different from procaine.

Discussion. In a preliminary report (see footnote on page 44) describing a few of these experiments, the tentative conclusion was drawn that the inhibiting effect of PABA and DEAE upon procaine convulsions can be best explained by the mechanism known as competitive inhibition.

There is apparently at least one alternative theory if one makes the unsupported assumption that the occurrence of convulsions is produced by the destruction of the procaine molecule. Since this is presumably a hydrolytic process catalyzed by a procaine esterase which has recently been studied in detail by Kisch (16) one could assume that—at least *in vitro*—the presence of the end-product of the hydrolysis, namely, PABA and/or DEAE, would tend to arrest further hydrolysis according to the law of mass action. There are numerous objections against this explanation. No evidence is available proving that *in vitro* conditions, under which such an action might occur, are duplicated *in vivo*. Furthermore, if such mechanism would be present, maximal inhibition should occur by simultaneous administration of a mixture of procaine with its split products. It will be remembered that this was not the case but optimal protection followed the previous injection of the split products. In other experiments, no increase in the convulsive action of procaine in guinea pigs was noted by administration of eserine which, according to Kisch (17), inhibits procaine esterase.

The lack of an unspecific anticonvulsive effect of PABA or DEAE has been demonstrated in the above described experiments with metrazol and Diothane.

Thus, the assumption of the presence of a competitive inhibition due to structural similarity of the convulsive agent and the inhibitor seems most plausible from the experimental evidence.

The results shown in table 1 indicate the relation between chemical structure and the degree of competitive inhibition. The importance of the para-configuration is shown by the complete absence of a protective action with orthoaminobenzoic acid. In accord with this is the moderate activity of para-hydroxybenzoic acid while again the correponding ortho-form (salicylic acid) is inactive. This latter compound is noticeably more toxic than the para form. Unfortunately, none of the meta analogues were available. Benzoic acid proved to be

the most efficient protector, except for PABA. It thus appears that any deviation from PABA itself weakens the protective action and that the change to an ortho configuration reduces this activity to a greater degree than removal of the NH_2 radical or its substitution by an OH group in para positions. Walker and Derow (18) recently reported an analogous condition in the field of microbiology, namely, the failure of ortho and meta aminobenzoic acid to show the well-known antagonism of PABA against the bacteriostatic effect of sulfonamides.

A close relation between the protective efficacy and chemical constitution is also evident with the aminoalcohols. DEAE itself could be expected to exert the greatest effect, but the increase by one CH_2 (No. 2) did not diminish the effectiveness when compared with DEAE itself, as long as the length of the straight chain of the alcoholic group remains unchanged. The isomeric compound (No. 3) with a straight chain is less active and no protective action is exerted by the next higher homologue (No. 4). No. 5 and 6 were inactive while No. 7, a secondary amine and isomeric to DEAE itself, had some activity. The substitutions of both ethyl groups in DEAE by propyl (No. 8) resulted in a marked increase of toxicity, which necessitated a considerable reduction of the dose, but a significant protective effect followed the administration of 1.5 mM/kg. However, here, as with No. 7, a definite depression was seen in the animals after the injection of procaine, suggesting a summation of the depressing action of both drugs. The reason for the activity of No. 8, ethyldiethanolamine is evident from its structural similarity to DEAE since three chains, one of them ethyl and two ethanols, are bound to the N atom, thus providing one more alcoholic group than in DEAE itself, but having the same number of carbon atoms arranged identically around the nitrogen as in DEAE. A consideration of the structures of these compounds reveals that in general, the presence of three chains consisting of at least two carbons on the nitrogen are necessary for activity but one butyl group instead of two ethyl groups will still preserve some activity. Thus, it is not necessary that the structure of a primary amine be preserved. All compounds had at least one primary alcohol group. An attempt to use triethylamine (as the hydrochloride) had to be abandoned on account of the marked depression and fatalities that followed the administration of doses equimolar to those of the aminoalcohols.

Antagonistic actions between drugs of similar structure, aside from the well-known example of PABA and the sulfonamides in the metabolism of bacteria, have been quoted by Clark (19); among those of interest are the antagonistic effect of higher members of the series $RN(CH_3)_3$ against the muscarine action of lower members, also the observation that ephedrine antagonizes certain effects of epinephrine. Ing (20) assumes that ephedrine attaches itself to the same receptors as epinephrine without exerting an action. According to this conception, graded responses result from the number of receptors occupied.

It is probable that a similar competitive antagonism is the modus operandi in these experiments. This is apparently the first report where a well-defined pharmacologic effect of a drug (ester) in an intact animal can be inhibited by the previous administration of either of its split products (alcohol or acid) or mixtures of the two which are themselves pharmacologically inert in the doses used.

The mechanisms and biochemistry of convulsions in general is still obscure and

little is known with regard to the particular convulsive effect of procaine. These limitations should be kept in mind in considering the schematic attempt to picture the mechanism of effects reported in this paper as shown in figure 1.

The "receptor" is pictured as the place in the CNS to which the drugs attach themselves. It is open to further investigation if this receptor is the cell surface, an enzyme, or a substrate. In this scheme, certain configurations are allotted to procaine and to its components. It is evident that the occupation by either the PABA or DEAE of one part of the receptor will prevent the attachment of a whole molecule of subsequently injected procaine. This conception takes into

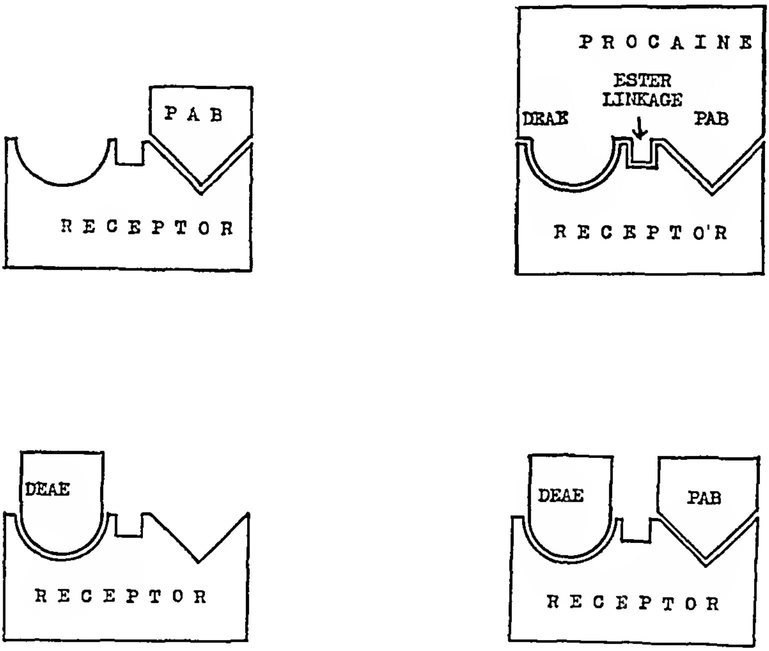

Fig. 1

consideration the observation that the injection of the mixture of PABA and DEAE not only fails to produce convulsions but exerts an even greater protective effect. The typical convulsive effect is elicited only by the whole procaine molecule, which possesses the ester linkage and attaches itself to the corresponding surface of the receptor. Obviously many other variations of the scheme can be devised or other assumptions made, as, for instance, a change in the physical chemical property (lipoid solubility) by the presence of the ester group which may result in intracellular penetration of the procaine itself or by other substances provided the cell surface is not already occupied by inert compounds, more specifically PABA or DEAE.

Accordingly, one could allot to the various derivatives of either PABA or DEAE

schematic configuration more or less similar to these two "specific" substances which would account for the graded effectiveness as protecting agents. Our results show that if the protective agents were injected before or simultaneously with the procaine, a greater number of moles of the procaine split products must be present than of procaine itself in order to prevent convulsions. This suggests that the receptors have a greater affinity to the whole molecule and it must be admitted that we do not know what forces constitute this property nor the laws that govern them. It is particularly in this aspect, where principal questions of this receptor theory arise.

Preliminary attempts to repeat such experiments in mice, golden hamsters or dogs were unsuccessful and have met with only slight success in rabbits. The failure in the first two species can be explained on the basis of their relatively great resistance to procaine, which necessitates the administration of extremely large doses of the inhibitors beyond the limit of their tolerances. The dog is not very tolerant to DEAE, which in toxic doses produces convulsions. PABA alone was not protective in the amounts in which it could be used. We can only speculate that the degree of "affinity" between the receptor and procaine or its split products is different in various species of animals, but numerous examples exist for qualitative or quantitative differences in action or antagonism of drugs in various species.

Aside from these considerations, we must take into account the difference in speed with which procaine is destroyed by various species. We have observed in our experiments in guinea pigs that the duration of the protective effect of PABA and DEAE is limited and it becomes to a certain degree a race between the rates of excretion of the protective agents and the destruction of procaine. This became evident in numerous animals where the pre-treatment delayed the onset of the convulsions but did not prevent their eventual occurrence.

Under the conditions of our experiments, no impairment of the peripheral local anesthetic effect of procaine by PABA or DEAE could be demonstrated. The reasons for this may rest in a quantitative difference rather than in a qualitative one unless one chooses to assume the presence of essentially dissimilar properties in the metabolism or excitability between the cell body and the dendrite. The presence of numerous synaptic connections in the CNS permits the speculation that the effects which we are discussing take place on these locations. If this is so, one must consider an effect upon the acetylcholine-esterase equilibrium which is concerned with synaptic transmission. Valasquez and co-workers (21) recently confirmed the potentiating effect of procaine upon acetylcholine by inhibition of the esterase and made the interesting observation that DEAE, but not PABA, exerts a similar effect.

Many of the challenging questions which arise from these observations remain unanswered at the present time and must be left to future investigation.

SUMMARY

It has been shown that the incidence of convulsions which follow intramuscular injection of procaine in guinea pigs can be substantially reduced by preceding administration of either of the split products of procaine; namely, para-aminoben-

zoic acid (PABA) or diethyl-aminoethanol (DEAE). A mixture of the two components is even more effective in this respect.

Certain derivatives of both PABA and DEAE possess qualitative similar action, the degree of which is dependent on their structural relations to the mother compounds. It has been shown that this protective action is specifically directed against convulsions produced by procaine and that no inhibition of peripheral local anesthetic action of procaine occurs.

Evidence has been presented to explain the phenomena observed in these experiments on the basis of competitive inhibition.

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STUDIES ON THE MECHANISM OF ACTION OF THIOUREA AND RELATED COMPOUNDS

I. Metabolic Changes after Acute Poisoning by Alpha-naphthylthiourea[1]

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Alpha-naphthylthiourea (ANTU) has recently been recommended (1) as a rodenticide for the extermination of Norway rats. The compound appears to be quite suitable for this purpose because it is very toxic to Norway rats and is much less toxic to dogs, cats, monkeys, and several other species.

In addition to its practical value as a rodenticide, ANTU is a useful compound for the study of some of the pharmacological problems which are common to other drugs and toxic substances. Species differences in susceptibility to the agent, the development of tolerance after administration of sub-lethal doses, and the higher resistance of young animals toward ANTU are marked characteristics associated with the toxicity of this thiourea derivative.

Richter (1) has studied the pathological changes resulting from the administration of lethal doses of ANTU to several species, and has found that intense pleural effusion and pulmonary edema were the most prominent and consistent pathological changes. ANTU is a useful compound for studying the mechanism underlying increased capillary permeability because of its pronounced action on the lung tissue together with the ease with which the compound can be administered and the dosage regulated.

No studies on the chemical changes in the blood and tissues from animals after acute ANTU-poisoning had been carried out prior to the recent preliminary investigation in this laboratory (2) in which it was found that ANTU produces hyperglycemia, a depletion of liver glycogen, and an inability to deposit liver glycogen in rats receiving a lethal dose of the compound.

The present communication contains the results of more extensive studies on the blood and tissue changes in dogs and rats after acute ANTU-poisoning.

The mode of action of this compound is of interest because of the possible wide use of ANTU as a rodenticide. Furthermore, findings with this compound may be of value in explaining the acute toxicity of some of the other thiourea derivatives which have recently attracted considerable attention because of their effects on the thyroid gland (3).

In these studies particular attention was directed to the changes in blood glucose at intervals after administration of various doses of ANTU. The results of

[1] This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

zoic acid (PABA) or diethyl-aminoethanol (DEAE). A mixture of the two components is even more effective in this respect.

Certain derivatives of both PABA and DEAE possess qualitative similar action, the degree of which is dependent on their structural relations to the mother compounds. It has been shown that this protective action is specifically directed against convulsions produced by procaine and that no inhibition of peripheral local anesthetic action of procaine occurs.

Evidence has been presented to explain the phenomena observed in these experiments on the basis of competitive inhibition.

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TABLE 1

Changes in plasma and blood constituents of dogs after poisoning by ANTU

Dog 1

75 mgm./kgm. ANTU

Survival time—20 hours

DETERMINATION	HOURS AFTER ANTU			
	0	4	7	11
Glucose (mgm. %)	96	145	146	150
Pentose (mgm. %)	6.2	6.7	8.0	7.4
Inorganic P (mgm. %)	4.7	6.3	6.2	6.5
Hematocrit (%)	45.0	59.0	60.0	65.0

Dog 2

100 mgm./kgm. ANTU

Survival time—8 hours

DETERMINATION	HOURS AFTER ANTU				
	0	0.3	1	2.5	4.0
Glucose (mgm. %)	100	143	330	360	283
Pentose (mgm. %)	7.5	9.2	17.0	20	15
Inorganic P (mgm. %)	5.0	5.3	5.7	5.5	6.0
Hematocrit (%)	40	58	65	69	76

Dog 3

100 mgm./kgm. ANTU

Survival time—6 hours

DETERMINATION	HOURS AFTER ANTU				
	0	0.6	2	3.5	5.5
Glucose (mgm. %)	100	190	143	133	100
Pentose (mgm. %)	4	6	5	5	5
Inorganic P (mgm. %)	4.5	4.5	4.5	4.7	5.5
Hematocrit (%)	40	45	46	48	50

Dog 4

150 mgm./kgm. ANTU

Survival time—8 hours

DETERMINATION	HOURS AFTER ANTU			
	0	2	6	8
Glucose (mgm. %)	92	290	50	50
Pentose (mgm. %)	5	19	8	10
Inorganic P (mgm. %)	4.5	4.6	5.4	15.5
Hematocrit (%)	41	48	57	70

these experiments indicate that ANTU produces a severe disturbance in carbohydrate metabolism in dogs and rats. Similar changes had not been observed previously in acute poisoning from this compound or from any of the other thiourea derivatives.

METHODS. Adult dogs and adult Sprague-Dawley albino rats were used in these studies. All of the animals were maintained on Purina Chow diet. ANTU was dissolved in propylene glycol with the aid of gentle heat. Five per cent solutions of ANTU in propylene glycol were injected intraperitoneally into dogs and solutions containing 5 mgm. of ANTU per cc. were administered by the same route to rats. The amounts of propylene glycol employed produced no toxic effects by themselves and caused no changes in the blood constituents which were studied.

Blood samples were taken by cardiac puncture from ether-anesthetized rats and by venepuncture from dogs. Heparin was used as the anti-coagulant. Whole blood from rats and plasma from dogs were used for the analyses.

Blood analyses included the following determinations: glucose, by the method of Folin and Malmros (4); calcium, by the method of Clark and Collip (5); magnesium, by the method of Briggs (6); protein and non-protein nitrogen, by the micro-Kjeldahl method as described by Koch (7); pentose, by the method of Mejbaum (8); and lactic acid, by the method of Barker and Summerson (9). Inorganic phosphorus was determined by the method of Fiske and Subbarrow (10) and acid-extractible organic phosphorus on 1:10 trichloracetic acid filtrates by the same method.

For glycogen determinations the animals were killed by decapitation, and the tissues were quickly removed and placed on dry ice. This procedure was completed within one minute to prevent glycogenolysis. Glycogen was determined by the method of Good et al. (11).

EXPERIMENTAL. *Toxicity of ANTU to dogs and rats.* The toxicity of ANTU to rats has been extensively studied by Richter (1) who found that the LD50 for laboratory rats was close to 4.0 mgm./kgm. when aqueous or oil suspensions of the drug were injected intraperitoneally. This investigator found, however, that there was considerable strain difference in toxicity of ANTU to rats which makes it desirable to determine the toxicity to every strain employed.

Our finding that ANTU is soluble in propylene glycol made it possible to employ solutions of the agent for injection. The approximate LD50 of ANTU for adult Sprague-Dawley rats was 5.0 mgm./kgm. when propylene glycol solutions were injected intraperitoneally.

Among the common laboratory animals dogs are second to rats in susceptibility to ANTU (1). The toxicity of propylene glycol solutions of ANTU for our adult dogs was determined prior to mechanism studies. Toxicity studies on 18 adult dogs gave an approximate LD50 value of 50 mgm./kgm. for this species.

Blood changes in dogs poisoned by ANTU. The effects of lethal doses of ANTU on the plasma constituents of dogs were determined by taking blood samples at intervals between the time of administration of ANTU and the time of death of the animals. These determinations were made in an effort to obtain information on the type of metabolic disturbance produced by this toxic agent. The results of these analyses are summarized in table 1. Hematocrit values, the intraperitoneal dose of ANTU, and the survival time for each animal are also given.

The chemical analyses of plasma samples from ANTU-poisoned dogs indicated

None of the dogs receiving doses of ANTU of 60 mgm./kgm. or less than that amount showed increases in plasma glucose comparable to those obtained with 10 mgm./kgm. in rats. Dog no. 7 receiving two times the LD50 dose (100 mgm./kgm.) showed an increase in plasma glucose similar to that seen in rats with 10 mgm./kgm. The blood glucose of two of the animals (dogs no. 14 and no. 15) remained below 90 mgm. % throughout the sampling period and these two animals survived. Thus, higher quantities of ANTU are necessary for dogs than for rats to produce hyperglycemia, and it appears that these changes are not evident when sub-lethal doses are given.

TABLE 2

Relationship between amount of ANTU, hyperglycemia, and survival time of dogs

DOG NO	ANTU	SURVIVAL TIME	PLASMA GLUCOSE (MGM %)			
			0 hours	0 5 hours	1 hour	3 hours
	mgm./kgm.					
7	100	6	61	193	193	285
10	60	20	61	87	105	115
11	60	22	74		97	133
12	60	23	60	95	97	107
8	50	14	70	97	105	130
9	50	16	65	90	120	
14	50	Survived	60		77	76
13	40	30	83	100	108	118
15	40	Survived	86	86	86	60

TABLE 3

Blood glucose, phosphorus, and pentose of ANTU-poisoned rats

RAT NO	BEFORE ANTU			2 5 HOURS AFTER ANTU (10 MGM /KGM)		
	Glucose	Phosphorus	Pentose	Glucose	Phosphorus	Pentose
	(mgm %)	(mgm %)	(mgm %)	(mgm. %)	(mgm. %)	(mgm. %)
1	73	7.5	19.0	146	10.5	14.7
2	66	6.5	15.0	160	11.0	20.7
3	70	7.5	16.0	219	7.0	19.0
4	68	5.0	16.4	176	8.0	16.8
5	67	5.7	14.6	176	11.0	14.0
6	66	6 7	17.0	160	9 4	21.8
Avg.	68	6.5	16 3	173	9 2	17.8

Blood changes in ANTU-poisoned rats. To compare the blood changes produced in rats with those observed in ANTU-poisoned dogs, glucose, pentose, and inorganic phosphorus were determined on blood samples taken before and after injection of 10 mgm./kgm. of ANTU. The results of these determinations are given in table 3.

There was a large increase in blood glucose of rats in 2.5 hours after adminis-

that this compound produces a marked interference with carbohydrate metabolism. In every case there was a rise in blood glucose occurring soon after the injection of ANTU. In dog no. 1, which received 75 mgm./kgm. of ANTU, the rise in glucose was smaller but more persistent than that observed in the other animals, and this animal survived the longest. Pronounced hyperglycemia, occurring within an hour after injection of ANTU, was seen in dogs no. 2 and no. 3 which received 100 mgm./kgm. of ANTU. Dog no. 4, which received 150 mgm./kgm. of ANTU, showed hyperglycemia followed by hypoglycemia in 6 hours after injection of the drug.

The rapid onset of hyperglycemia was somewhat surprising in view of the low solubility of ANTU in aqueous and oil solutions together with the fact that it is a relatively slow acting poison with death rarely occurring within 6 hours after administration of lethal doses.

All of the animals showed a rise in plasma pentose which was greatest and occurred earliest in dog no. 4. In dog no. 2 an increase of 12.5 mgm.% was observed while in dogs no.1 and no. 3 a much smaller increase in pentose occurred. There was some increase in inorganic phosphorus in all of the samples from poisoned animals. However, both the changes in inorganic phosphorus and pentose occurred after the changes in blood glucose. All of the animals showed hemoconcentration, the increase from 41 to 70% in dog no. 4 being the largest increase observed in these experiments.

In addition to the determinations listed in table 1 other analyses were carried out on the same plasma samples. However, none of these other plasma constituents showed any marked variation from normal in any of the animals throughout the sampling period. Thus, plasma calcium values rémained between 11 and 14 mgm. %, and there was no significant change in magnesium from the average normal value of 1.80 mgm. %. Plasma chloride did not deviate significantly from the normal of 382 mgm. %, nor did plasma protein from the average normal value of 5.6 grams % for these animals. There was an increase in non-protein nitrogen at 8 hours after ANTU in dog no. 1 from a normal of 29 mgm. % to 51 mgm. %. However, there was no significant rise in the non-protein nitrogen of plasma in any of the other animals.

Plasma lactic acid was determined on two other dogs (dogs no. 5 and no. 6) after administration of 75 mgm./kgm. of ANTU intraperitoneally. In dog no. 5 the lactic acid rose from 6.2 to 11.0 mgm. % then fell to 8.4 mgm. % in 7.5 hours. Dog. no. 6 showed an increase from 1.6 mgm. % to 8.3 mgm. % in 1.5 hours and this value fell to 6.4 mgm. % in 7.5 hours after ANTU.

Relationship between amount of ANTU and glucose changes in dogs. Previous studies in this laboratory (2) have shown that 10 mgm./kgm. of ANTU will cause pronounced hyperglycemia in 2.5 hours in rats. It was of interest to compare the dose necessary to produce hyperglycemia in the dog with that necessary to produce a similar change in rats, which are considerably less resistant to this compound. The extent of the glucose changes in dogs receiving lethal and sublethal doses of ANTU as well as the survival time after various doses of ANTU was determined. The results of these experiments are shown in table 2.

None of the dogs receiving doses of ANTU of 60 mgm./kgm. or less than that amount showed increases in plasma glucose comparable to those obtained with 10 mgm./kgm. in rats. Dog no. 7 receiving two times the LD50 dose (100 mgm./kgm.) showed an increase in plasma glucose similar to that seen in rats with 10 mgm./kgm. The blood glucose of two of the animals (dogs no. 14 and no. 15) remained below 90 mgm. % throughout the sampling period and these two animals survived. Thus, higher quantities of ANTU are necessary for dogs than

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tration of 10 mgm./kgm. of ANTU. The rise was comparable to that obtained in dogs (table 1) with much larger doses. There was an increase in inorganic phosphorus in all except rat no. 4, and some increase in pentose in 4 of the animals. The results indicate that the same carbohydrate changes take place in ANTU-poisoned rats and dogs, but the dose required to produce these changes is less in rats, to which the compound is considerably more toxic. The amount of ANTU required to produce similar blood changes bears a close relationship to the LD50 in the two species.

Effect of ANTU on the glycogen of rat tissues. The hypoglycemia which sometimes followed hyperglycemia in dogs after administration of large doses of ANTU suggested the possibility that liver glycogen was being depleted. This possibility was tested in rats receiving 10 mgm./kgm. of ANTU intraperitoneally. Glycogen determinations were also made on skeletal muscle and cardiac muscle from normal and poisoned animals. The normal animals were fasted for 12 hours before death and the ANTU-poisoned animals were fasted 6 hours before ANTU

TABLE 4

The effect of ANTU on the glycogen content of rat tissues

RAT NO.	CONTROL			RAT NO.	ANTU-POISONED (10 MGM./KGM.)		
	Heart	Skeletal muscle	Liver		Heart	Skeletal muscle	Liver
	%	%	%		(%)	(%)	(%)
1	0.20	0.18	3.5	5	0.23	0.07	0.12
2	0.41	0.40	2.3	6	0.26	0.09	0.12
3	0.21	0.20	2.1	7	0.29	0.18	0
4	0.24	0.23	2.5	8	0.13	0.07	0
				9	0.15	0.15	0
Avg.......	0.27	0.25	2.5		0.21	0.11	0.05

and for 6 hours after ANTU and were then sacrificed for glycogen determinations. The results of these determinations are given in table 4.

Liver glycogen showed the greatest decrease from 2.5% in the normal animals to 0.05% in the poisoned group. This depletion of liver glycogen probably accounts for the hypoglycemia which was sometimes observed (table 1) when large doses of the toxic agent were given. There was also some decrease in skeletal muscle glycogen, but there was no significant lowering of the glycogen of cardiac muscle.

Effect of insulin on ANTU-poisoning in dogs. Attempts were made to prevent the rise in glucose by administration of insulin to ANTU-poisoned dogs. One dog (dog no. 16) received 2 units/kgm. of insulin subcutaneously and 100 mgm./kgm. of ANTU intraperitoneally at the same time. In 1 hour the blood glucose increased from 90 to 200 mgm. % but in 3 hours it fell to 106 mgm. %. The other animal (dog no. 17) received 2 units of insulin/kgm. and in 3 hours when the blood glucose had fallen to 50 mgm. % then 100 mgm./kgm. of ANTU was given

intraperitoneally. In one hour after the administration of ANTU the blood glucose had risen to 88 mgm. % and in 4 hours to 144 mgm. %. Insulin was effective in postponing the onset of hyperglycemia in dog no. 17, but did not prevent death. Dog no. 16 survived for 6 hours and dog no. 17 survived for 14 hours after ANTU.

Effect of cysteine on ANTU poisoning. From the chemical structure of ANTU it seemed possible that this compound might react with sulfhydryl groups of tissue constituents. We had previously found (2) that 1000 mgm./kgm. of cysteine given intraperitoneally 5 minutes after ANTU would prevent death from an LD100 dose. In this connection, it was of interest to determine how soon the cysteine must be given in order to exert its protective effect. Therefore, rats were given 7 mgm./kgm. of ANTU (an LD100 dose for these animals) and then were given 1000 mgm./kgm. of cysteine at various intervals after ANTU. For injection cysteine hydrochloride was dissolved in distilled water and neutralized with sodium hydroxide. The solution contained 100 mgm. cysteine/cc. The results of this experiment are given in table 5.

TABLE 5

Effect of cysteine on the toxicity of ANTU to rats

7 mgm./kgm. ANTU; 1000 mgm./kgm. Cysteine; 5 rats/group

GROUP	TIME OF CYSTEINE AFTER ANTU	% MORTALITY	GROUP	TIME OF CYSTEINE AFTER ANTU	% MORTALITY
	hours			*hours*	
1	control	100	6	3	0
2	0.1	0	7	4	0
3	0.5	0	8	5	20
4	1	0	9	7	100
5	2	0			

Cysteine was effective when given within 5 hours after ANTU but would not protect the animals if given 7 hours after injection of the toxic compound. It is interesting that lung damage begins to appear about 5 hours after administration of lethal doses of ANTU to rats, and it appears that cysteine was ineffective in reversing this effect. The same amount of cysteine was ineffective against lethal doses of ANTU in dogs. However, the lethal dose of ANTU for dogs is much higher than that for rats and it seems probable that much higher doses of cysteine would be necessary to counteract the effects in this species. Such a high dose of cysteine might be toxic in itself and thus limit the usefulness of this substance in counteracting ANTU poisoning.

Effect of adrenal cortical extract on ANTU poisoning. In connection with the carbohydrate changes observed in these experiments it was of interest to test the effect of adrenal cortical extract on the toxicity of ANTU to dogs. One dog (dog no. 18) was given 1 cc. of Lipo-Adrenal Cortex (Upjohn) intramuscularly 1.5 hours before ANTU (100 mgm./kgm.), 1 cc. at 0.5 hours after ANTU and 3 subsequent doses of 1 cc. each at intervals of 2 hours. The plasma glucose in-

creased to 160 mgm. % but returned to normal in 2 hours after injection of ANTU. The animal survived for 23 hours.

Another dog (dog no. 19) received 2 cc. of Lipo-Adrenal Extract during the 2 hours immediately preceding injection of 100 mgm./ kgm. of ANTU and then received 1 cc. of the extract intramuscularly at two-hour intervals for 8 hours. The blood glucose increased from 100 mgm. % to 216 mgm. % in 1 hour after ANTU and then fell to 66 mgm. % in 5 hours. This animal survived for 16 hours.

While there was some increase in the survival time and the hyperglycemia was of shorter duration than in untreated animals, adrenal cortical extract did not prevent the lethal action nor the symptoms produced by 100 mgm./kgm. of ANTU in dogs.

Discussion. The change in the blood glucose after intraperitoneal injection of ANTU in both dogs and rats was the most marked and consistent metabolic change observed in ANTU-poisoned animals. The extent of hyperglycemia increased with the dose administered, and there was a correlation between the species susceptibility to the toxic substance and the amount of ANTU required to produce changes in blood glucose. In our experience with dogs it has been possible to predict whether the animals will survive by following the plasma glucose values after the administration of ANTU. In animals which survive the glucose did not rise appreciably throughout the sampling period.

The appearance of hyperglycemia soon after the administration of this toxic substance indicates that at least part of the injected dose rapidly enters thd circulation in spite of the low solubility of this compound in aqueous and oil solutions.

Less significance is attached to the rise in inorganic phosphorus which always occurred later than the rise in glucose in ANTU-poisoned animals and may be secondary to the changes in glucose. The rise in pentose was not a consistent finding in all of the poisoned animals.

The increase in hematocrit is attributed to the loss of plasma resulting from the increased permeability of the blood vessels of the lungs.

The fall in blood glucose sometimes to sub-normal levels can be explained by the depletion of liver glycogen. The blood glucose changes and the depletion of liver glycogen could be attributed to an inhibition of glycolysis and/or respiration by ANTU. The rise in glucose would, thus, result from the inability of poisoned tissues to utilize glucose. An alternate explanation involves the possibility of increased glycogenolysis in ANTU-poisoning. At the present time this possibility seems the more likely, especially since insulin was partially effective in preventing the hyperglycemia. However, the exact mechanism by which these changes are produced remains to be investigated. In this connection, the possible role of adrenalin in producing these changes seems worthy of investigation.

The effectiveness of cysteine in preventing death from lethal doses of ANTU in rats indicates that ANTU can react with sulfhydryl groups. That cysteine is effective if given as long as 4 hours after ANTU does not necessarily imply that

this antidote has reversed a reaction between ANTU and some cellular constituent because the low solubility of ANTU makes it possible that several hours may elapse before the total injected dose is absorbed. Thus, cysteine, even when given 4 hours after ANTU, might combine directly with ANTU and in that way effectively decrease the amount of ANTU capable of combining with tissue constituents.

The results of these experiments demonstrate metabolic changes produced by ANTU which had not previously been described for this compound or for any of the other thiourea derivatives. Since the pathology resulting from the acute toxicity of thiourea and mono-substituted derivatives of thiourea is very similar (12, 13, 14) it seems likely that metabolic changes similar to those described here for ANTU will be found to result from acute poisoning by other thiourea derivatives.

SUMMARY

1. Determination of the toxicity of alpha-naphthylthiourea (ANTU) for rats and dogs gave an LD50 of 5.0 mgm./kgm. for rats and 50 mgm./kgm. for dogs by intraperitoneal injection.

2. Lethal doses of ANTU produced a rise in plasma glucose, phosphorus, and pentose in dogs and rats, and the dose required to produce the changes varied with the susceptibility of the species to ANTU.

3. There was an increase in hematocrit and in blood lactic acid of ANTU-poisoned dogs.

4. No change in plasma calcium, magnesium, nitrogen, chloride, and acid-soluble organic phosphorus was observed in ANTU-poisoned dogs.

5. Liver glycogen of rats fell from the average normal value of 2.5% to 0.05% in 6 hours after administration of 10 mgm./kgm. of ANTU. There was some decrease in skeletal muscle glycogen but no change in glycogen of heart muscle.

6. Insulin was partially effective in decreasing the extent and duration of hyperglycemia. Adrenal cortical extract showed some beneficial effect in ANTU-poisoned dogs.

7. Cysteine (1000 mgm.) prevented death of rats receiving an LD 100 dose of ANTU (7 mgm./kgm.), if the cysteine was given within 5 hours after ANTU.

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THE ACTION OF 1,1-DIPHENYL-1-(DIMETHYLAMINOISOPROPYL)-BUTANONE-2, A POTENT ANALGESIC AGENT[1]

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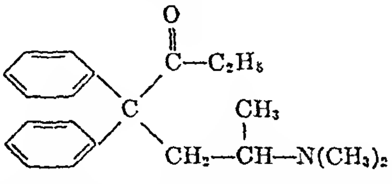
In spite of numerous attempts to find new analgesic compounds superior to morphine or other opium alkaloids, little progress was made until 1939 when Eisleb and Schaumann (1, 2) reported the action of dolantin (demerol), a substance producing both morphine-like and atropine-like effects. Following the Allied victory of World War II, knowledge of additional related compounds, prepared by German chemists, became available through a team headed by Dr. Ervin C. Kleiderer, Technical Industrial Intelligence Committee under the Foreign Economic Administration, U. S. Department of State. A report by the Committee has been published by the U. S. Department of Commerce (3). Of the many compounds disclosed, 1,1-diphenyl-1-(dimethylaminoisopropyl)-butanone-2 appeared to be outstanding. This compound bears the German serial number 10820. Since details of pharmacological work were not available in the above-mentioned report, we undertook an investigation of the actions of 10820. The following is an account of our studies. Drs. E. Rohrmann and T. P. Carney, of our organic chemical department, prepared the compound.

For our experimental work, the hydrochloride salt of 10820 was employed. It is a white crystalline compound, soluble in water and alcohol, but insoluble in ether. It has a bitter taste and melts at 236–236.5°C. The structural formula as given by the Germans is as follows:



For brevity, the product will be referred to below as a butanone derivative or by its original German number 10820.

1. Action on the nervous system. *Analgesic action.* Albino rats were injected intraperitoneally with various amounts of 10820. Tests for analgesia were made by the tail-pinching technique of Haffner (4). The threshold dose for analgesia was found to be 1 mg. per kg., lasting 1 to 2 hours; whereas 10 mg. of demerol per kg. of body weight was the smallest dose to produce analgesia in the same animals.

More detailed studies were made in 7 trained unanesthetized dogs. The pain

[1] Read at the Atlantic City meeting of the American Society for Pharmacology and Experimental Therapeutics on March 12, 1946 (11).

threshold was determined by the Wolff-Hardy method (5). The shaved skin of the back served as the area of exposure to the light beam, the end-point being a skin-twitch, as suggested by Andrews and Workman (6). Considerable training of these animals was necessary to obtain a constant basal threshold and to prevent conditioned responses. The smallest dose of 10820 which produced a definite rise in the pain threshold was found to be 1 mg. per kg. Figure 1 shows a comparison of the analgesic effects of this compound and morphine. A dose of 5 mg. of 10820 per kg. of body weight resulted in marked analgesia. Larger doses caused even greater effects, raising the threshold to a point where the skin

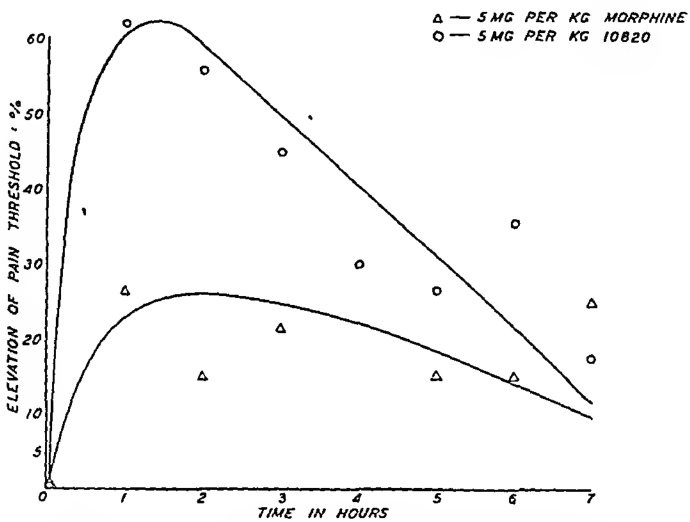


FIG. 1. COMPARISON OF THE ANALGESIC EFFECTS OF 10820 AND MORPHINE SULFATE IN UNANESTHETIZED DOGS

Pain thresholds were measured by the Wolff-Hardy method. Each curve is the average for 7 dogs. Administration was intraperitoneal.

was severely burned. Weight for weight, 10820 appeared to be somewhat stronger than morphine sulfate.

Other effects of this substance on the nervous system of dogs closely resembled those of morphine. During the peak of analgesia, marked sedation was present. In addition, there was salivation and ataxia. When walking, the dogs usually assumed a hyenoid posture. Unlike morphine, 10820 never caused vomiting. Effects noted on the autonomic nerves will be discussed later.

Analgesic action in 3 male adults was tested, also utilizing the Wolff-Hardy apparatus. The forehead blackened with grease paint was the area of exposure. Following thorough training, the threshold without medication remained practically constant. Orally, 5 mg. of 10820 caused definite analgesia. In all 3 subjects, this dose was compared with 150 mg. of demerol, also taken by mouth.

Figure 2 shows the results in 1 subject, similar effects occurring in the other 2 individuals. It is obvious that on a weight basis 10820 is several times more analgetic than demerol.

Certain side-effects may be noted following ingestion of the drug. In 12 subjects given 5 mg. (total), a lightheaded feeling (not true vertigo) was noted by 50 per cent of the individuals. This dose may cause mild sedation also. One person developed slight nausea and sweating about 1 hour after taking the drug. Side-effects occurred in only 20 per cent of the persons who received 2.5 mg. orally. These symptoms were similar to those resulting from 150 mg. of demerol. In addition, the latter drug caused dryness of the mouth, an effect not present with 10820. The pulse rate usually dropped 5 to 10 per minute, and the blood pressure fell a few millimeters of Hg, an action which was probably due to sedation. No change occurred in respiratory rate at this dose level.

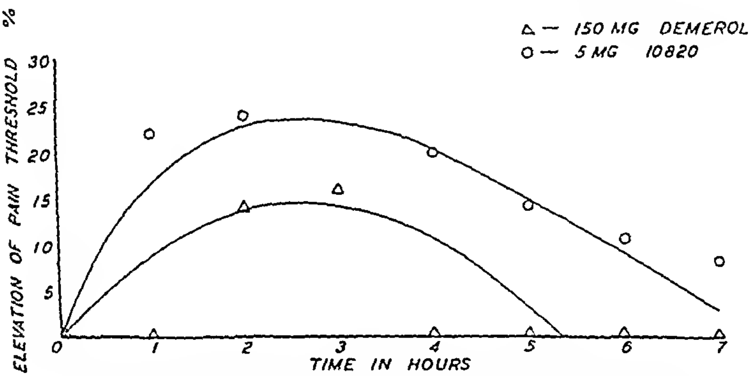


FIG 2 ANALGESIC ACTION OF 10820 IN A HUMAN ADULT MALE
Pain was produced by thermal radiation of a blackened spot on the forehead Comparison was made with demerol, both compounds being taken by mouth.

2. MORPHINE-LIKE ACTION IN MICE, RATS, AND CATS Mice injected intraperitoneally with 10820 showed a typical Straub reaction of the tail which could not be distinguished from that seen following morphine administration. Eisleb and Schaumann (1) noted the same phenomenon with demerol.

Rats characteristically developed marked postural rigidity, accompanied by coma, as a result of injections of this compound. The degree of both effects increased with the size of the dose Following large doses, the rats appeared to be almost dead. Respiratory depression became so marked that considerable cyanosis developed. The animals would remain in this state for 2 to 4 hours before onset of recovery. Similar results occurred with demerol and morphine.

The nervous excitation of cats typically seen following morphine also resulted from 10820 This consisted of nervousness, wild, purposeless running, salivation plus respiratory stimulation, and convulsive twitches. These findings occurred a few minutes after subcutaneous injection. In about 1 to $1\frac{1}{2}$ hours the

animals became depressed, but crawled around in a swimming manner with all 4 extremities extended and abducted. One cat given 10 mg. of the compound per kg. of body weight remained in this depressed state and odd posture for 2 days before it recovered. Morphine appeared to produce greater stimulation (mania) than 10820, and lacked the peculiar depressive effect of the latter.

3. EFFECTS ON CIRCULATION AND RESPIRATION. (*A*) *Anesthetized dogs.* Under barbiturate anesthesia, blood pressure, respiration, and kidney volume were recorded by the usual techniques. In addition, the volume of expired air was quantitatively measured (7). A typical record is shown in figure 3. Intravenous injection of 10820 in the dose of 2 mg. per kg. produced temporary apnea followed by marked respiratory depression. This dose usually caused a decrease of about 50 per cent in the volume of expired air. Respiratory depression lasted

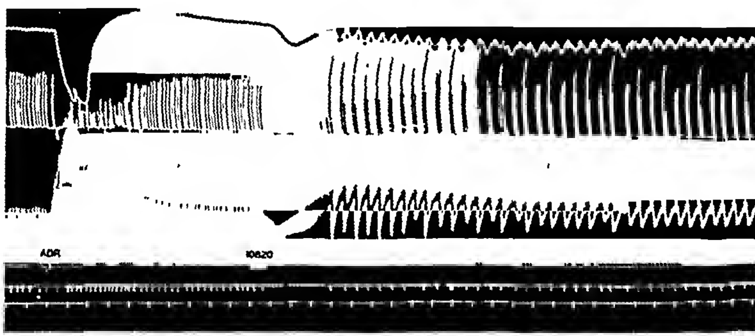


FIG. 3. ACTION OF 10820 ON CIRCULATION AND RESPIRATION

A dog, weighing 15 kg., was anesthetized with sodium phenobarbital. From above down, the recordings are kidney volume, respiratory tambour, carotid blood pressure, 0 mm. Hg, expired air in 500 cc. volumes, and 1-minute intervals. At ADR, 1 cc. of 1:10,000 epinephrine hydrochloride was injected intravenously. Dose of 10820 was 2 mg per kg. intravenously, resulting in marked cardiac slowing.

1 to 2 hours, and longer with larger doses. Results were quite similar to those that occurred with morphine. Demerol, on the other hand, caused only slight changes in respiration.

A temporary moderate fall of blood pressure and a decrease of kidney volume followed intravenous injection of the drug. This suggested a decreased cardiac output. Most striking, however, was a marked bradycardia which persisted many minutes. The cardiac slowing appeared to be mainly due to excitation of the vagus nerves, since it was abolished by atropine. Furthermore, after section of the vagi, 10820 caused only slight decrease of heart rate. Unlike demerol, the substance did not antagonize the action of acetylcholine. Thus, it appeared that 10820, like morphine, has parasympathomimetic properties. Morphine and demerol produced much more marked and prolonged depression of the blood pressure than this butanone derivative.

(*B*) *Unanesthetized dogs.* Experiments were performed with 7 dogs trained to lie quietly on a table while pulse and respiratory rates were counted or electrocardiograms recorded. Their initial average pulse rate was 76 per minute, and after intraperitoneal injection of 10820, 5 mg. per kg., it dropped to 43 per minute. The mean respiratory rate before the drug was 20 per minute, and, at the height of action, it was 14 per minute. In a similar test with morphine sulfate, 5 mg. per kg. caused the pulse rate to fall from 61 to 42 per minute and depressed respirations from 19 to 16 per minute. The maximal action on pulse or respiratory rate occurred anytime from 1 to 7 hours after administration of either substance, and the depressive effects lasted many hours. It appears that the 2 drugs caused almost identical changes in pulse and respiratory rates.

Electrocardiograms were taken because of the possibility that A-V block or some other abnormality might have produced the slow heart rate. However, aside from very occasional premature systoles, electrocardiograms showed only sinus bradycardia with prolongation of the whole PQRST complex. This effect resulted from hyperactivity of the vagus nerves, since injection of atropine sulfate was followed promptly by tachycardia. A similar action with morphine sulfate was reported by van Egmond (8).

4. Salivary secretion. In anesthetized dogs salivary secretion was recorded by cannulation of Wharton's duct, the cannula being connected to a drop recorder. Salivary flow was stimulated by pilocarpine. A slight decrease in the drop rate could be demonstrated by intravenous injection of 10820 in the dose of 2 mg. per kg. Atropine and demerol promptly caused complete cessation of salivary flow.

Trained unanesthetized dogs showed an entirely different salivary response to this compound. A dose of 2 mg. per kg. intraperitoneally produced salivation within a few minutes which often lasted several hours. Salivation could be quickly inhibited by atropine, so the stimulation appeared to be parasympathetic in origin.

5. Action on intestines. When tested on the isolated small intestines of rabbits, 10820 showed an antispasmodic action. The intestinal segments were suspended in Locke-Ringer's solution by the usual method of recording, and stimulated to contract by mecholyl. The activity of the butanone compound was about equal to that of demerol, but only 1/200 as great as that of atropine sulfate.

Further studies were made on trained unanesthetized dogs[2] having an exteriorized skin-covered intestinal loop ("suit-case handle") as reported by Wakim and Mason (9). The reaction of the animal to the drug depended somewhat on the level of small intestine from which the record was taken. A dose of 5 mg. (total) intravenously produced stimulation of motility of the ileum of 15 to 45 minutes' duration. Typical records are seen in figure 4. Thereafter, intestinal movements appeared to be depressed. In the duodenum and jejunum, the period of stimulation from 10820 was shorter, the inhibiting effect of the compound

[2] These dogs were prepared by Dr. K. G. Wakim, Indiana University Medical School, Bloomington, Indiana.

being more prominent. In practically every trial, defecation occurred at some time, once or several times. These reactions were similar to those obtained with morphine. They probably were the result of parasympathetic stimulation, as in the heart and salivary glands. Of interest was the fact that during the analgesic tests in dogs, gurgling sounds were frequently heard several hours after administration of either morphine or the butanone compound.

6. Tolerance studies in dogs. Intraperitoneal injections of 10820 were made daily for 28 consecutive days into the 7 trained dogs mentioned above. The dose was 1 mg. per kg. for the first week and 2 mg. per kg. thereafter. After a rest period of 1 week, the same procedure was repeated with 5 mg. per kg. of morphine sulfate, except that the duration was only 22 days. At weekly intervals, analgesic action was measured by the Wolff-Hardy method. In addition, observations were made of side-reactions. As shown in figure 5, no tolerance developed to the analgesic effect of 10820. Whereas the dogs became definitely

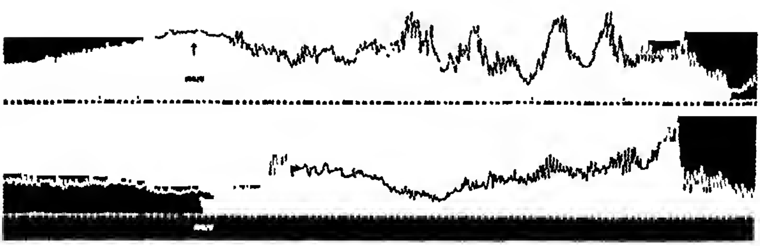


Fig. 4. Action on Small Intestines of 2 Unanesthetized Dogs

Records are of motility of skin-covered loops of intestine, the upper record indicating the movements of the lower ileum, and the lower tracing, those of the upper ileum. The preliminary motility was the normal response to feeding. Dose of 10820 was 5 mg. total intravenously. Time intervals were 10 seconds.

more tolerant to morphine, their susceptibility to the butanone derivative appeared to increase. This change was progressive from week to week and did not vary in direction. Recently, Goetzl, Burrill, and Ivy (10) found that their dogs became tolerant to the analgesic action of morphine but tolerance was not lasting, tending to disappear and reappear. We did not encounter this phenomenon, although our study was of shorter duration than theirs. Concerning side-reactions, a similarity existed between the two drugs. At the end of the experimental periods, the animals showed distinctly less sedation, ataxia, and salivation. In the case of morphine, no tolerance occurred to its emetic action. Vomiting never resulted from administration of 10820. Loss of appetite as indicated by a progressive decrease of body weight resulted with both compounds.

7. Toxicity. (*A*) *Acute toxicity in mice.* The median lethal dose ± standard error of 10820 was determined by intravenous administration into mice. Calculations were made by the method of Bliss (11). Simultaneously, a determi-

nation was made of the toxicity of demerol. For 10820 and demerol, the LD_{50} was 20.56 ± 1.35 and 49.67 ± 1.35, mg. per kg., respectively. Thus, the former was only about 2.4 times as toxic as demerol, although the butanone compound was several times as potent analgetically.

(*B*) *Chronic feeding experiments in rats.* Young rats weighing around 100 grams were fed diets containing 10820 or demerol in various percentages (12). Six groups of 5 rats each were tested on each compound. The dose ranged from 0.01 to 0.5 per cent of the food for the newer substance, while with demerol the range was from 0.02 to 1.0 per cent. The test lasted 28 days, at the end of which time all surviving rats were sacrificed for necropsy.

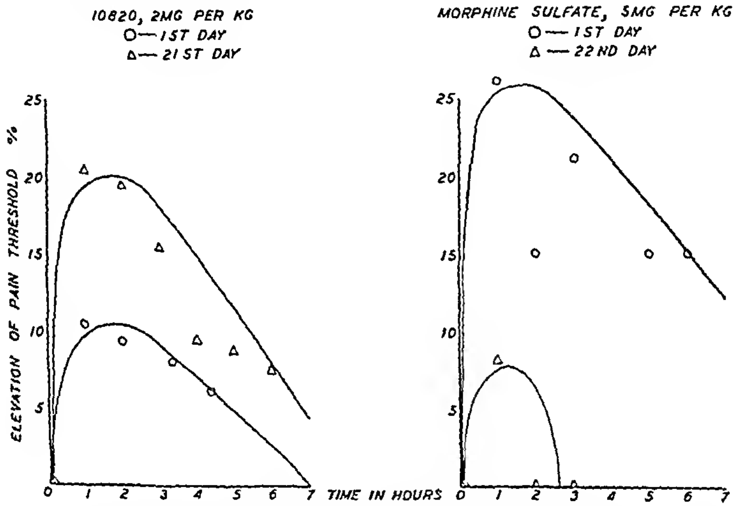


FIG. 5. TOLERANCE STUDIES IN TRAINED DOGS

Intraperitoneal injections of 10820 were made daily for 28 consecutive days. The dose was 1 mg. per kg. the first week and 2 mg. per kg. thereafter. The procedure was repeated with morphine sulfate, 5 mg. per kg., for 22 days. Each curve is the average for 7 dogs. Note the tolerance to morphine, while the dogs apparently became more susceptible to the analgesic action of 10820.

Weight gain appeared to be depressed somewhat by all concentrations of 10820 and demerol. However, there was no difference in the degree of depression produced by the former in concentrations of 0.2 per cent or under, while with 0.5 per cent, growth was distinctly less. Amounts of demerol up to 0.5 per cent were tolerated equally, while 1.0 per cent caused a more marked inhibition of weight gain. There were no deaths except in the groups which received the 2 highest doses of each drug. The 0.2 and 0.5 per cent diets of 10820 killed 1 and 2 rats, respectively. Death occurred in 1 and 4 rats receiving, respectively, 0.5 and 1.0 per cent concentrations of demerol. As with the LD_{50}, the butanone derivative was approximately 2.5 times as toxic as demerol.

Necropsy findings appeared to be non-specific. Many of the rats were entirely normal. With both compounds, slight hypertrophy of the thyroid gland was

observed. Interstitial pneumonia was rather common, but was more frequent in animals fed the smaller amounts. On the 0.5 per cent dose of 10820, 2 animals showed myocardial necrosis and 1 rat had fatty metamorphosis of the liver. The same changes were seen in 1 rat on the 0.2 per cent diet of this substance. Both the cardiac and liver changes, however, were focal and minimal.

(C) *Effect on red and white blood cells.* During the tolerance test of 10820 on the 7 trained dogs, red and white blood cell counts and hemoglobin determinations were made on the 14th day. Results were entirely negative. On the 28th day, white cell counts were still normal, and since hemoglobin values were unchanged, red cell counts were not done.

At the end of the chronic feeding experiments, red and white cell counts and hemoglobin values were ascertained in all rats surviving the 2 highest doses of 10820. Here again no change from the normal was found. This was true also of the rats fed the 2 highest doses of demerol. It appears that repeated administration of this butanone compound is without effect on the hematopoietic system.

8. Excretion in man. By modification of the method of Lehman and Aitken (13), excretion studies were made with 10820 in the urine of 3 normal adult males. The analytical procedure was adapted to the Beckman photoelectric spectrophotometer, the readings being taken at 625 $\mu\ \mu$. Because the dose of the drug was so much smaller than that of demerol, vacuum distillation of urine samples to produce a 10-fold concentration was usually necessary. On an oral dose of 5 to 7.5 mg., about 20 to 35 per cent was excreted over a period of 24 hours. Lehman and Aitken found about 5 to 20 per cent of an ingested dose of demerol in the 24-hour urine specimens. The fate of the unexcreted 10820 was not ascertained.

9. Clinical results. Dr. K. G. Kohlstaedt, of the Lilly Laboratories for Clinical Research, has kindly allowed us to include a preliminary report of his trials at the Indianapolis City Hospital. A total of 30 patients received the compound for pain of various etiology. In most instances, the dose was 5 mg. orally every 3 or 4 hours, although doses of 2.5 mg. were used also. Pain was effectively relieved in 80 per cent of all cases. The group was too small to make conclusions, but some of the results were of particular interest. In all 5 patients with malignant tumors, pain was alleviated. Postoperative pain was adequately controlled in 8 of 11 individuals. In 2 cases of gangrene, no relief occurred. Analgesic action was noted also in myalgia, dysmenorrhea, headache, and toothache.

Nausea was the most frequent side-action. It occurred in 9 patients at some time during the course of medication. In 4 cases, vomiting was associated with nausea. Headache and dryness of the mouth were noted 4 and 2 times, respectively. One patient had a lightheaded feeling and pallor. No severe side-actions were encountered. In some instances, patients preferred to continue medication in spite of untoward effects.

SUMMARY

A study was made of the actions of 1,1-diphenyl-1-(dimethylaminoisopropyl)-butanone-2. In rats, dogs, and man, it possesses marked analgesic action, being

at least equal to morphine and several times more potent than demerol. In many respects this substance is closely similar to morphine. However, qualitative differences between the effects of morphine and this butanone derivative have been noted. Apparently, there is little or no tolerance development to the analgesic action of 10820 in dogs. Side-reactions in human beings do not appear to be excessive. Clinical results so far substantiate the laboratory data.

Acknowledgments: The authors wish to acknowledge help from a number of individuals. Thanks are due to Dr. P. N. Harris who performed the necropsies, and Messrs. E. B. Robbins, F. W. Israel, and R. C. Anderson and Miss Nila Maze for their invaluable assistance in many experiments.

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PHARMACOLOGY OF LOBELAN AND RELATED COMPOUNDS

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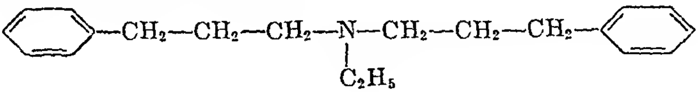
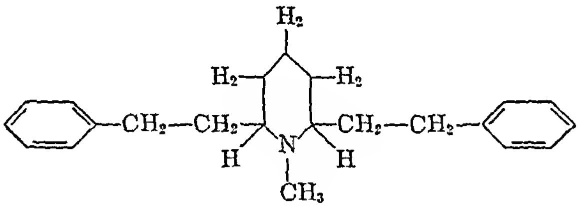
Many types of chemical substances produce a paralzying effect on smooth muscle, either directly, through the nervous mechanism, or both. In the search for more efficient spasmolytics, Kulz and Rosemmund (1) introduced di-(γ-phenylpropyl)-ethylamine (Sestrone).



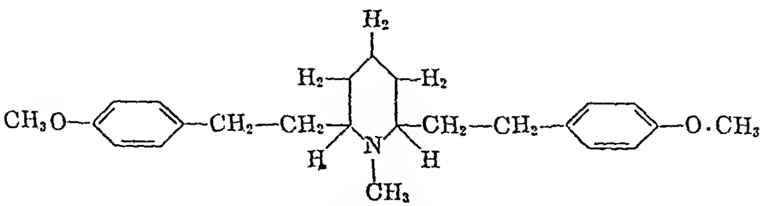
Sestrone

Lee and Freudenberg (2) discussed the literature dealing with the spasmolytically active di-(phenylalkyl)-alkylamines. In considering further development along these lines, they synthesized lobelan,



Lobelan

and other di-2,6-(phenylaklyl)-piperidines and also derivatives of di-2,4-(phenylalkyl)-piperidine and 2-phenylalkyl-4-alkylpiperidine. The most efficient spasmolytic of the series was found to be compound Ih,



Compound Ih

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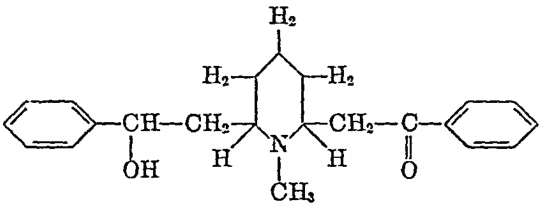
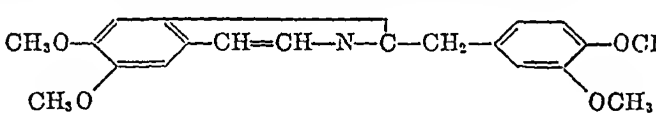
Lobelan is the parent base of the lobeline series of alkaloids from Lobelia inflata. It has apparently not been hitherto pharmacologically examined but lobeline



Lobeline

and other closely related compounds such as lobelanin and lobelanidin have been extensively examined particularly for respiratory stimulating activity (3). Camp (4) in a rather cursory study observed that lobeline (alpha lobeline) had a relaxing action followed by contraction on dog intestine in situ. This is apparently the only observation in the literature concerning a possible spasmolytic action of the lobeline series. Our findings that lobelan and its derivatives possess potent spasmolytic action together with Camp's observations suggest that the entire lobeline series may have spasmolytic action. The structure of papaverine, a highly potent spasmolytic agent, if written differently than in the usual manner is seen to bear a resemblance to the lobelan series, viz:



Papaverine

Because of the structural similarity of the lobelan series to lobeline respiratory stimulating action would be expected by the former and this anticipated action is realized. This paper is a report of some of the pharmacological effects of the compounds prepared and reported by Lee and Freudenberg.[1]

Toxicity. The LD50 was determined in mice by subcutaneous and intravenous injection of the hydrochlorides of the bases. The data are given in tables 1 and 2. Because of low solubility of the hydrochloride the ethane sulfonate salt of compound Ih was used in later work. The subcutaneous LD50 of this salt is 450 mg./kg. (N = 140) and the intravenous LD50 17.3 mg./kg. (N = 75). By the subcutaneous route it is much less toxic than the hydrochloride, the LD50 of the latter being 250 mg./kg. (N=60). The difference in molecular weights (478 and 403 respectively) does not account for the lower toxicity.

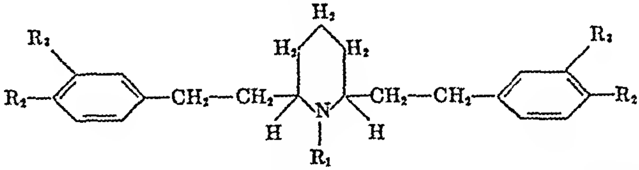
The subcutaneous LD50 in the rat was 975 mg./kg. (N = 100) and 17.5 mg./kg. (N = 22) in the rabbit. The intravenous LD50 on the rabbit was 5

[1] Compounds have been labeled to correspond to the designations used by Lee and Freudenberg.

mg./kg. (N = 14) and the oral LD50 112 mg./kg. (N = 14). These doses are all for the ethane sulfonate of Ih.

Papaverine·HCl was also tested in rats and rabbits. The rat subcutaneous LD50 was 620 mg./kg. (N = 92). The rabbit intravenous LD50 was approximately 25 mg./kg. (N = 2). Additional mouse tests yielded somewhat higher

TABLE 1



COMPOUNDS (HCl SALTS EMPLOYED)				LD50 IN MICE		SPASMOLYTIC ACTION§	RESPIRATORY ACTION‖ PER CENT INCREASE IN MINUTE VOLUME
No.	R_1	R_2	R_3	Subcutaneous	Intravenous	Conc.	
				mg./kg.*	mg./kg.†		
Ic	H	H	H	325	24	100×10^{-8}	252
Ia‡	CH_3	H	H	250	15	53×10^{-8}	175
Id	C_2H_5	H	H	425	27	133×10^{-8}	55
Ie	C_3H_7	H	H		31	133×10^{-8}	
If	C_2H_4OH	H	H			80×10^{-8}	
Ig	H	OCH_3	H	75	21	33×10^{-8}	750
Ih	CH_3	OCH_3	H	250	18	27×10^{-8}	220
Ii	C_2H_5	OCH_3	H	90	18	27×10^{-8}	
Im	C_2H_4OH	OCH_3	H		17	67×10^{-8}	
Il	H	O—CH_2—O		115		60×10^{-8}	−30
Ik	H	OH	OCH_3	350		670×10^{-8}	20
Papaverine·HCl				350	23	167×10^{-8}	62
Atropine·½H_2SO_4·H_2O						2.7×10^{-8}	

* N = 25 to 60.
† N = 5 to 17.
‡ = Lobelan.
§ = Concentration producing moderate relaxation of guinea pig intestine stimulated with acetylcholine chloride 2.7×10^{-8}.
‖ = 5 mg./kg. morphine and 2 mg./kg. of drug, both intravenously.

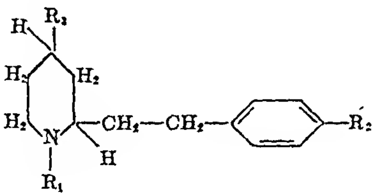
figures than given in the table, namely 440 mg./kg. (N = 80) by the subcutaneous route and 28./kg. (N = 20) by the intravenous route.

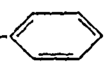
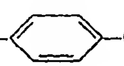
Action on smooth muscle. Spasmolytic tests were conducted on isolated tissues suspended in aerated Tyrode solution at 37°C. With guinea pig intestine antagonism to acetylcholine chloride is shown by the results averaged in tables 1 and 2. The most active compounds were Ig, Ih, and Ii, whose concentrations were, respectively, 33, 27, and 27 $\times 10^{-8}$; that of papaverine·HCL was 170 $\times 10^{-8}$.

Using guinea pig uterus stimulated with 1×10^{-4} barium chloride, compounds Ia, Id, IVb and IVd were effective in concentrations of 133, 133, 133, and 667 $\times 10^{-8}$, respectively, and papaverine·HCl at 1000×10^{-8}. However, in a later experiment compound Ih·HCl and papaverine·HCl, each at a concentration of 400×10^{-8}, were effective against $BaCl_2$ 1.3×10^{-4}.

Since compound Ih seemed to be the most efficient, additional tests were made with the ethanesulfonate salt. In table 3 are given the concentrations of this

TABLE 2



COMPOUNDS (HCl SALTS EMPLOYED)				LD50 IN MICE		SPASMOLYTIC ACTION§	RESPIRATORY ACTION‖ PER CENT INCREASE IN MINUTE VOLUME
No.	R_1	R_2	R_3	Subcutaneous	Intravenous	Conc.	
				mg./kg.*	mg./kg.†		
IVb	CH_3	H	$CH_2 \cdot CH_2$— 	100	14	233×10^{-8}	95
IVe	C_2H_5	H	$CH_2 \cdot CH_2$— 	125	22	53×10^{-8}	160
IVc	C_2H_5	OCH_3	$CH_2 \cdot CH_2$—  —OCH_3	>300	32	133×10^{-8}	
IVa	C_2H_5	H	H	65	5	267×10^{-8}	
IVd	CH_3	CH_3	H	125	18	267×10^{-8}	161

* N = 10 to 42, except IVc, where N = 3.
† N = 9 to 20.
§ and ‖ see footnotes table 1.

compound, papaverine and atropine effective against acetylcholine, barium and histamine. Typical results are shown in figures 1 to 4.

Using the method of Sollmann and von Oettingen (5) on isolated perfused guinea pig lungs, compound Ih (hydrochloride) offered some antagonism against the bronchoconstrictor effect of simulataneously administered histamine. It was at least 20 times less effective than epinephrine. Results were not quantitative, but approximately the effect of a dose of 0.02 mg. of histamine dihydrochloride was inhibited by 0.01 mg. of epinephrine or 0.2 mg. of compound Ih. In the unstimulated lungs, compound Ih (0.5 mg.) caused a moderate increase in the rate of perfusion.

Respiratory-stimulating activity. The effect of respiratory stimulation was studied in rabbits depressed with 5 mg./kg. of morphine sulfate administered intravenously. The compounds were also injected intravenously at a dose of

TABLE 3

Concentrations of spasmolytic agents producing a moderate rate of relaxation of guinea pig intestine

STIMULANT	CONCENTRATION	Ih ETHANE SULFONATE	PAPAVERINE HYDROCHLORIDE	ATROPINE SULFATE
Acetylcholine chloride	4×10^{-8}	36×10^{-8}	500×10^{-8}	1×10^{-8}
Histamine dihydrochloride	20×10^{-8}	60×10^{-8}	400×10^{-8}	200×10^{-8}
Barium chloride	2×10^{-4}	500×10^{-8}	400×10^{-8}	1500×10^{-8}

TABLE 4

Per cent increase in minute volume of respired air in rabbits which had received 5 mg./kg. of morphine sulfate intravenously

SUBSTANCE	INTRAMUSCULAR DOSE	AVERAGE[1] % INCREASE IN MIN VOL FOR 2 HOURS
	mg./kg	
Lobeline	10	23.3
Metrazol	25	19.4
Ih (ethane sulfonate)	10	19.2
Saline	1 cc.	−3.7

[1] N = 5 for each drug.

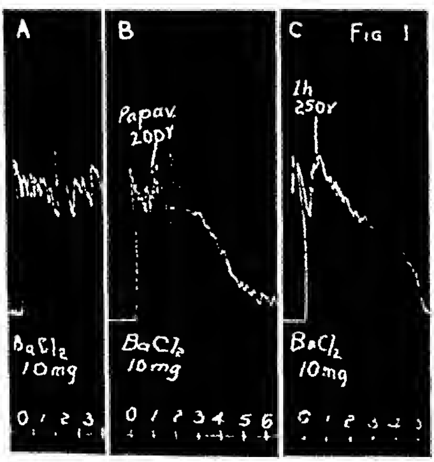


FIG. 1. BARIUM STIMULATED GUINEA PIG INTESTINE
Bath. Tyrode Solution, 50 cc
Lower line time in minutes

A. Control
B. 200 γ papaverine hydrochloride
C. 250 γ Ih ethane sulfonate

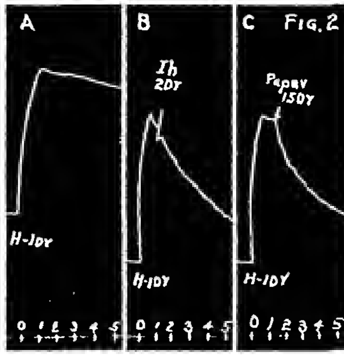


Fig. 2. Histamine Stimulated Guinea Pig Intestine

A. Control
B. 20 γ Ih ethane sulfonate
C. 150 γ papaverine HCl

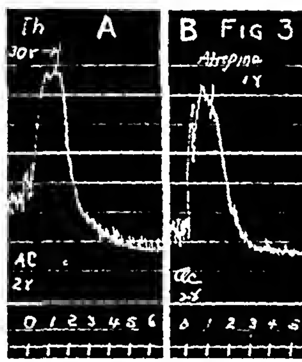


Fig. 3. Acetylcholine Stimulated Guinea Pig Intestine

A. 30 γ Ih ethane sulfonate
B. 1 γ atropine sulfate

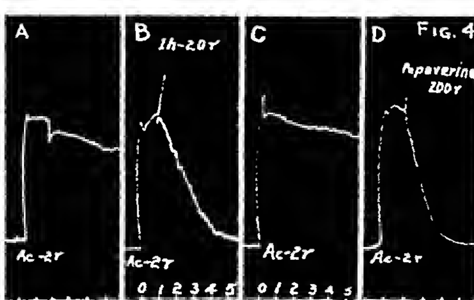


Fig. 4. Acetylcholine Stimulated Guinea Pig Intestine

A. Control
B. 20 γ Ih ethane sulfonate
C. Control
D. 200 γ papaverine HCl

2 mg./kg. and the volume of respired air measured by means of a spirometer and recorded on a kymograph. The minute volume was calculated from the tracings. Tables 1 and 2 give the per cent increase in minute volume in single animals.

Additional work was done with compound Ih (ethanesulfonate salt) using intramuscular injections. The morphine was given intravenously as before. The increase in minute volume rose gradually and then tapered off over a period of two hours. Figures were calculated representing the average per cent increase in minute volume of respired air over the two hour period. Five rabbits were used with each drug, and the average values given in table 4. The averages do not reflect the observation that metrazol and lobeline gave greater initial stimulation but that compound Ih gave a more uniform stimulation throughout the two hour period.

Antagonism to allylisopropyl barbituric acid (alurate). Rats in groups of 20 were injected subcutaneously with 40 mg./kg. of the sodium salt of allylisopropyl barbituric acid. Thirty minutes later compound Ih, lobeline, or benzedrine was administered. The doses were respectively, 5 and 10, 5, and 0.5 and 1.0 mg./kg. The sleeping times were significantly shortened in all cases except with one 0.5 mg./kg. dose of benzedrine. Except for this one case the differences in awakening action were small.

Life saving action was studied on mice. 200 mg./kg. of the sodium salt produced an 80% mortality. This dose was administered to mice in groups of 5 or 10 animals. At intervals of ½, 2, and 6 hours, doses of compound Ih were given in doses of 25, 50, and 100 mg./kg. subcutaneously. In no case was the mortality significantly altered.

Circulation. All of the compounds caused a fall in blood pressure. With 1 mg doses in ether-anesthetized cats, the fall was usually 15 to 20 mm. Hg. However, compound Ia caused a fall of 30 mm. Hg and its isomer compound IVb a fall of 55 mm. of Hg. Recovery was rapid and second doses 15 to 20 minutes later caused similar effects.

The effect on the heart rate was slight; there was either slowing or no change. This variability was most apparent with compound Ih, and in two experiments the slowing was marked rather than slight. It was abolished by atropine.

Symptomatology. Rabbits. As intravenous doses of compound Ih were increased progressively to the fatal level there first appeared signs of restlessness, excitement and muscular tremors. These symptoms were succeeded by incoordination and occasional slight spasms. The highest doses usually produced convulsions and death occurred either during or following a convulsion. Animals surviving convulsions were invariably prostrate for an hour or more. The respiratory rate was always stimulated. With oral doses of the drug the results were similar though more variable.

Mice. Subcutaneous injections caused signs of marked central stimulation. In addition to convulsive seizures with high doses, salivation was present. Tail erection and exophthalmos generally occurred. As with rabbits, prostration succeeded the convulsive stage.

Discussion. The compounds studied bear a structural resemblance to both papaverine and lobeline and the predictions that they would have both spasmolytic and analeptic properties are borne out by the data presented.

Compound Ih usually caused relaxation without loss of rhythmic contractions and occasionally the latter were temporarily increased in amplitude. Papaverine invariably caused relaxation with a gradual or abrupt diminution of the rhythmic contractions.

Winterstein (6) in a general review of the lobeline alkaloids cited the suggestion that lobeline might be of use in bronchial asthma. Compound Ih should possess greater antiasthmatic action than lobeline, but it seems unlikely either drug would be especially efficient.

In regard to the relation between structure and spasmolytic activity, the introduction of a methoxy group in the p-phenyl position is most important. In the compounds of series I (table 1), it invariably enhances the action. However, if one phenethyl radical is in the 4-piperidine position the addition of p-methoxy reduces the spasmolytic activity, (cf. compounds IVe and IVc). Shifting the 6-phenethyl group to the 4-position on the piperidine ring reduces the activity except for compound IVe found to be more potent than its analog Id. Substitution of methyl for the 4-phenethyl (compound IVd) has no effect on the activity, but replacement by hydrogen (compound IVa) greatly reduces the activity.

In general, as structural chances are made, toxicity parallels the spasmolytic activity. However, compound Ih possessing about the same spasmolytic activity as compounds Ig and Ii is only one-third as toxic by subcutaneous administration as the latter.

SUMMARY

1. Piperidine compounds of the lobelan type have been studied for spasmolytic action, respiratory and analeptic action, blood pressure, and toxicity and were shown to possess both peripheral spasmolytic action and central stimulating action. The spasmolytic tests were conducted on isolated tissues.

2. The neurotropic action as studied on acetylcholine-stimulated guinea pig intestine was greater than that of papaverine for most of the compounds. The most efficient compound, 1-methyl-2,6-di-(p-methoxyphenethyl)-piperidine (compound Ih), was 10 to 14 times as effective as papaverine.

3. The myotropic action as studied on barium-stimulated guinea pig intestine was about the same order as that of papaverine. However, against histamine contractions, compound Ih was 7 to 10 times more powerful than papaverine.

4. Analeptic action was noted for the compounds. As a respiratory stimulant in morphinized rabbits, compound Ih was much more effective than as an antagonist against allylisopropyl barbituric acid.

5. The circulatory effects were relatively slight.

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THE SECRETION OF PANCREATIC JUICE IN THE PRESENCE OF ATROPINE OR HYOSCYAMINE IN CHRONIC FISTULA DOGS

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The effect of atropine on the secretion of pancreatic juice has been studied extensively in acute experiments on anesthetized animals. In general the results indicate that in these preparations atropine does not modify the response of the pancreas to HCl in the intestine (1) or to intravenous secretin (2). Savich (3) found that the secretory response to soap in the intestine was practically abolished in dogs by large doses of atropine (20–25 mgm. total dose). It is obvious that experiments on anesthetized animals can have only a limited significance in a situation in which the occurrence of reflexes is in question, as in the present instance.

Smirnow (4) studied the effect of atropine on the pancreatic secretion produced in response to fat in the intestine in a chronic fistula dog. The volume of juice was unaffected by atropine but the nitrogen content was reduced.

Bylina (5) studied the effect of atropine on the pancreatic secretion evoked by fats, soap or HCl in two chronic fistula dogs. He observed a decrease in total N, total solids and enzymatic activity of the juice after administration of atropine, regardless of the stimulus. When the stimulus was soap or HCl the volume of secretion was also reduced by atropine Babkin and Ishikawa (6), on the other hand, found that atropine (5 mgm , total dose) abolished the secretory response of the pancreas of a chronic fistula dog to neutral fat or oleic acid in the intestine. Soap still caused some secretion after atropine but the volume, and concentration of enzymes and total solids were reduced.

Tonkich (7) noted an increase in volume and a decrease in total nitrogen of secretion elicited by milk and butter in the intestine after giving atropine. She attributed the secretory response to some constituent of milk other than fat since fats alone were without much effect in her animals

It has been amply demonstrated that atropine in adequate dosage abolishes the secretory effect on the pancreas of stimulating the vagus nerves but the minimal dose required has, apparently, never been determined. Pavlov (8) and also Savich (3) used 20 mgm. (total dose) but the size of the dogs was not stated. Modrakowski (9) demonstrated paralysis of the pancreatic vagus after 0 8 mgm. per/kgm of atropine in an acute experiment Smaller doses were not tried. Von Anrep (10) obtained no secretory response to stimulation of the vagus after 0.36 mgm /kgm. in an acute experiment but he thought the "inhibitory" fibers were still functioning

Several investigators (9, 11, 12) have noted a secretagogue action of atropine on the pancreas when given in massive doses (10–80 mgm./kgm.).

The influence of atropine on the secretory response of the pancreas to products

of protein digestion in the intestine has not been studied. This problem is of special interest because peptone evokes a secretion which closely resembles that obtained on stimulation of the vagus nerves (13). We undertook this investigation primarily for the purpose of studying this problem; we also took advantage of the opportunity to add to the few observations that have been made on the effect of atropine on the response of the pancreas to other stimuli in chronic fistula dogs.

METHODS. Thirteen chronic fistula dogs and eleven anesthetized dogs were used in this study. Each of the chronic dogs had a tubulated fistula of the duodenum placed opposite the main pancreatic duct. All had gastric fistulas. Three had modified Thiry loops of the duodenum or upper jejunum. The Thiry loop was modified by anastamosing the distal end to the side of the loop near the proximal end thus providing for circulation of the contents of the loop.

Pancreatic juice was collected either through a small soft-rubber funnel inserted through the duodenal fistula tube and held against the mucosa surrounding the papilla (14), or through a temporary glass cannula inserted into the duct via the duodenal fistula as suggested by Scott and his co-workers (15) and later used by Hart and Thomas (16). Identical results were obtained by the two methods.

The dogs were fed once daily and were used for experiments before feeding when the stomach was empty. A drain tube inserted into the stomach through the gastric fistula prevented the passage of fasting gastric secretion into the duodenum. The inner end of the drain tube was held in the pyloric antrum by means of a thread passed through the pylorus and tied to the duodenal fistula tube.

Secretion of pancreatic juice was induced by injecting at intervals into the upper intestine either 20 cc. of a 5 per cent solution of commercial peptone (Bacto-protone), or 10 cc. of a 2 per cent solution of soap (Ivory Snow), or 10 cc. of N/10 HCl, or by giving secretin intravenously. The secretin was prepared in the laboratory and was at least equal in potency and purity to the commercial secretin ("Pancreotest") manufactured by Astra (Sweden) (17). It contained no vasodilators and no cholecystokinin.

During an experiment the stimulating substance was administered once every eleven to thirteen minutes and the pancreatic juice collected for ten minutes following each injection. The stimulus was administered at such intervals from four to six times and the subsequent "ten minute samples" collected during a control period after which the drug was administered and the experiment continued in the same way as long as necessary to establish clearly the character of the response. The volume and specific gravity of each sample were recorded. Total nitrogen was determined on some of the samples and on a smaller number enzyme determinations were made. Total nitrogen was calculated for other samples on the basis of a known linear relation[1] between specific gravity and total nitrogen of dog's pancreatic juice (18).

Atropine Sulfate (U.S.P., P.W.R.) was used in our early experiments. It was administered intravenously or intramuscularly in doses ranging from 0.2 to 0.3 mgm. per kilogram of body weight. In this dosage atropine causes excitement and the dogs frequently became unmanageable. We, therefore, changed to hyoscyamine hydrobromide (U.S.P. X, Merck) which was given in doses ranging from 0.1 to 0.2 mgm./kgm. No difference could be detected in the effects of the two drugs on pancreatic function but hyoscyamine seemed to cause less excitement. It was used in most of the experiments. The identity of action on the pancreas was to be expected since atropine is considered to be a racemic mixture of hysocyamine and its dextrorotatory isomer. The laevorotatory compound, used in these experiments, is believed to be mainly responsible for the peripheral effects (19).

Eleven acute experiments and two experiments on one of the chronic animals were done to determine the effect on the pancreatic vagus of the doses of hyoscyamine or atropine that we had used. For the acute experiments the dogs were anesthetized with 2 or 4 per cent chloralose in 20 per cent urethane given intravenously. The 4 per cent chloralose gave

[1] Total N (mgm./cc.) = 594.8 (Sp. g.—1.0075).

better anesthesia. About 2 cc. of the mixture per kilogram were required but more was given when necessary. The vagus nerves were stimulated in the neck with condenser discharges at a frequency of about 3 per second. This type of stimulation generally caused only moderate slowing of the heart. Pancreatic secretion was recorded graphically by means of a drop recorder. In a few instances faradic current from a Harvard coil was used and in two of these the nerves were stimulated below the heart. In all but five of the experiments the pylorus was closed by means of a submucous ligature. Ligature of the pylorus or differences in the method of stimulation did not affect the results in any consistent way except that manipulation of the pylorus tended to decrease the response of the pancreas to stimulation of the vagi whether or not a ligature was used.

In six of these dogs (nos. 22–27, table 1) one vagus was cut aseptically at least 10 days before the experiment and the other used for stimulation. Degeneration of one vagus appeared to enhance the response of the pancreas to stimulation of the other nerve and we

TABLE 1

Effect of hyoscyamine (or atropine) in the dosage used in this study on the response of the pancreas to electrical stimulation of the vagus

DOG NO.	WT.	VOLUME OF PANCREATIC JUICE SECRETED IN RESPONSE TO VAGUS STIMULATION							
		Control		After hyoscyamine			After physostigmine (following hyosc.)		
		Duration of stimulus	Volume	Dose	Duration of stimulus	Volume	Dose	Duration of stimulus	Volume
	kgm.	*min.*	*drops*†	*mgm./kgm.*	*min.*	*drops*	*mgm./kgm*	*min.*	*drops*
18	11	8	15	0.15	15	1	0.09	15	2
19	11	13	11	0.15	15	1	0.15	15	6
21	18	18	10	0.019	15	0			
21	18			0.15	15	0			
22	13	17	9	0.077*	15	2			
22	13			0.23*	15	0			
24	6	10	8	0.2	15	0	0.12	15	0‡
25	10	15	23	0.2	15	0	0.2	15	0
26	9	10	59	0.2	15	4	0.2	10	30
27	11	15	12	0.06	11	5			
27	11			0.15	15	3			

* Atropine sulfate.

† The dropper delivered 13 drops per cc. of distilled water. A displacement system was used, so arranged that only distilled water issued from the dropper.

‡ No response to secretin at this time.

think the procedure merits further trial in studies of this sort. In two of the five dogs with normal vagi (nos. 17 and 20) no secretion was obtained at any time in response to nerve stimulation. Dog no. 23 (one vagus degenerated) died before the experiment could be completed. Hence eight successful experiments were performed on anesthetized dogs.

In one of the chronic fistula dogs the left vagus was cut and chronically exposed according to the method of Pavlov (20) and the degenerating nerve stimulated on the fourth and fifth postoperative day. The experiment on the fourth day was done without anesthesia but on the fifth day the animal was so excitable that an anesthetic was necessary. He was given sufficient "Pentothal sodium" intravenously to cause muscular relaxation.

RESULTS. *The dose of hyoscyamine or atropine required to paralyze the pancreatic vagus.* The effect on the pancreas of stimulating the vagus nerves before and after administering hyoscyamine or atropine in anesthetized animals is shown in table 1. It is evident that in the dosage generally used in this study

these drugs effectively block the secretory endings of the vagus. Smaller doses were sometimes equally effective but in one instance at least the full dose of 0.2 mgm./kgm. of hyoscyamine failed to cause complete paralysis. The residual response (about 7.0% of the control) is practically negligible but serves to indicate that the doses used are within the range of the minimal effective dose for vagus paralysis. Because of the well known unreliability of the pancreatic secretory response to stimulation of the vagus nerves there is danger of mistaking failure of the response due to other causes for paralysis due to drugs. For this reason we attempted to restore the response in some animals by administering physostigmine sulfate after hyoscyamine paralysis. In three of five such experiments the response was partially restored by physostigmine.

TABLE 2*

Effect of atropine or hyoscyamine on pancreatic secretion in response to peptone injected into duodenum in normal dogs

DOG NO.	NUMBER OF EXPERIMENTS	CONTROL PERIOD (AVERAGES)			30–40 MIN. AFTER GIVING DRUG		
		Vol.	Sp g.	Total N	Vol	Sp g.	Total N
		cc		mgm /cc.	cc.		mgm./cc.
2-39	1	4.0†			12.0†		
3-39	1	2.6†			4.0†		
2-40	3	2.86	1.0178	6.1	1.73	1.0129	3.3
7-43	5	1.99	1.0170‡	5.65‡	11.1	1.0116	2.4
1-44	5	1.47	1.0224	8 8	2.98	1.0142	4.0
2-44	2	2.15	1.0183	6.45	2.85	1.0137	3.7
3-44	2	1.6	1.0186	6.5	1.15	1.0148	4.3
5-44	1	1.7	1.0177	6.0	3.8§	1.0106	1.8
1-45	2	2.7	1.0209	7.14	9.9¶	1.0114	1.89
2-45	2	2.25	1.0199	7.0	1.7	1.0119	2.66

* In this table and in tables 5, 6, and 7, the control values are averages for all samples collected during the control periods of all pertinent experiments on any one dog. The values given under "30–40 min. after giving drug" are averages of values for a group of single samples, one from each experiment, collected during the ten minute interval nearest the time indicated.

† Volumes estimated from graphic records.

‡ From 4 experiments.

§ 20–30 min. sample; volume fell off later as dog became excited.

¶ Includes one experiment terminated at end of 30 min.

The results obtained in experiments on the degenerating vagus in the chronic animal were similar to those obtained in anesthetized dogs. On the fourth post-operative day stimulation of the degenerating nerve with a faradic current (Harvard Coil, secondary at 8½ cm.) for 10 minutes caused the pancreas to secrete 2.5 cc. of juice of high specific gravity (1.0214). After giving 0.15 mgm./kgm. of hyoscyamine 0.2 cc. of juice was secreted during an identical period of stimulation. On the following day between 1.0 and 1.5 cc. of juice was obtained during each 10 minute period of stimulation. No secretion was obtained on repeating the stimulation immediately after giving 0.075 mgm./kgm. of hyoscyamine. Thirty minutes later the same stimulation produced 0.4 cc. of juice.

Changes in the response to Peptone. Twenty cc. of 5 per cent peptone in the intestine as used in the experiments on chronic animals normally causes secretion of about 2 cc. of highly concentrated pancreatic juice in 10 minutes. After giving atropine or hyoscyamine in the dosage used the volume of the ten minute samples characteristically *increased* while the specific gravity and total nitrogen invariably decreased. The increase in volume of secretion occurred consistently in 7 of the 10 normal dogs studied. In one dog (3-44) atropine or hyoscyamine caused no significant change in the volume of secretion and in two (2-40 and 2-45) the volume was decreased. The results are summarized in table 2.

TABLE 3

Control experiments with peptone injected into modified Thiry loop

DOG NO	NUMBER OF EXPERIMENTS	AVERAGE VOLUME OF CONSECUTIVE SAMPLES NOS 2-8						
		2	3	4	5	6	7	8
		cc.	cc	cc	cc	cc	cc.	cc.
4-44	4	1.3	1.05	0.95	0 65	1 1*	1.0†	0.2†
2-45	2	0 55	0 6	0.75	0.6	—†‡	—†‡	0.6†
5-45	6	0.98	0 86	0 95	0 86	0 98	0 78§	0 76¶

* Two experiments.
† One experiment.
‡ No data (cannula blocked).
§ Five experiments
¶ Three experiments.

TABLE 4

Effect of atropine or hyoscyamine on pancreatic secretion in response to peptone injected into modified Thirty loop

DOG NO	NUMBER OF EXPERIMENTS	CONTROL PERIOD AVERAGES			AFTER GIVING DRUG VOLUME OF SAMPLES NOS 1-4*			
		Vol	Sp g	Total N	1	2	3	4
		cc		mgm /cc	cc	cc	cc	cc
4-44	1	0 9	1 0198	7 35	0	few drops	0 2	few drops
2-45	3	0 61	1.0263	11 42	0.23	0 4	0.26	0 3†
5-45	3	1 16	1 0202	7 51	0 16	0 1	>0 1	few drops

* Corresponding in time to samples 5, 6, 7, and 8 in table 3.
† One experiment only.

When the peptone was injected into a modified Thiry loop of the duodenum or jejunum instead of the intact intestine, atropine or hyoscyamine completely abolished the secretory response or reduced it to such an extent that the residual secretion could not be distinguished from the spontaneous secretion which appears intermittently in normal dogs This result was obtained consistently in the three animals studied. Control experiments on these animals are summarized in table 3 and experiments with drugs in table 4.

Changes in the response to soap. Ten cc. of two per cent soap solution in the intestine causes an abundant secretion of pancreatic juice having a higher con-

centration of solids than that obtained with secretin or HCl but less than that obtained with peptone. Atropine or hyoscyamine caused a decrease in volume and specific gravity of the secretion. The specific gravity was lowered in some instances to the level characteristic of secretin juice. The results are summarized in table 5.

Changes in the response to HCl or secretin. Although these agents normally provoke secretion of relatively dilute pancreatic juice in terms of enzymes atropine or hyoscyamine generally caused a decrease in concentration as indicated by measurements of specific gravity. In a few experiments these changes were confirmed by nitrogen determinations and enzyme studies. The volume of secretion

TABLE 5

Effect of atropine or hyoscyamine on pancreatic secretion in response to soap in the intestine

DOG NO.	NUMBER OF EXPERIMENTS	CONTROL PERIOD (AVERAGES)			30–40 MIN. AFTER GIVING DRUG		
		Vol.	Sp.g.	Total N	Vol.	Sp.g.	Total N
		cc.		*mgm./cc.*	*cc.*		*mgm./cc.*
7-43	2	2.85	1.0146	4.2	0.5	1.0101*	1.5*
1-44	2	4.77	1.0151	4.5	3.4	1.098	1.4
1-45	2	6.8	1.0123	3.9	2.9	1.0102	1.28

* From one experiment only.

TABLE 6

Effect of atropine or hyoscyamine on pancreatic secretion in response to HCl in the intestine

DOG NO.	NUMBER OF EXPERIMENTS	CONTROL PERIOD (AVERAGES)			30–40 MIN. AFTER GIVING DRUG		
		Vol.	Sp.g.	Total N	Vol.	Sp.g.	Total N
		cc.		*mgm./cc.*	*cc.*		*mgm./cc.*
7-43	2	5.37	1.0109	2.0	2.0	1.0090	0.85
1-44	2	5.48	1.0117	2.5	3.6	1.0101	1.5
1-45	2	12.5	1.0087	0.63	7.1	1.0087	0.45

likewise was reduced by the drugs. The decrease in volume was greater when the stimulus was HCl than when secretin was being used. One animal (No. 3-44) showed no change in quantity of secretion obtained in response to secretin when hyoscyamine was given. This was the dog referred to in the section dealing with peptone as showing no change in volume of secretion. No experiments were obtained with soap or HCl in this animal and only one experiment was done with secretin. Tables 6 and 7 are summaries of experiments with HCl and secretin respectively.

Control experiments. More than one hundred experiments without drugs, in which the same technique was followed and the same stimuli used as in the experiments with atropine or hyoscyamine, have been performed on thirty normal dogs including those used in this investigation. A survey of these experiments,

particularly those which were continued for as long or longer than the experiments with drugs shows that no consistent changes in the quantity or quality of the pancreatic secretion occurred as a result of the passage of time or of repeated stimulation, except with soap. When soap was used the volume of the later samples tended to be somewhat greater than that of the early ones and specific gravity slightly less. Periodic changes, apparently associated with hunger periods, were noted in the response of the pancreas to each of the stimuli used. These comprised an increase in volume and, often, in specific gravity of the juice. All these changes were moderate and could not possibly be confused with the more pronounced effects of the drugs. These control experiments are distinct from the observations made during the "control period" which preceded each experiment with drugs. The latter were introduced to detect any abnormailty in the response of the pancreas to the specific stimulus on the day of the experiment.

Discussion. Administration of atropine or hyoscyamine in doses sufficient to paralyze the secretory endings of the vagi reduces the specific gravity and total nitrogen of pancreatic juice in the unanesthetized dog regardless of the stimulus

TABLE 7

Effect of atropine or hyoscyamine on pancreatic secretion in response to secretin given intravenously

DOG NO.	NUMBER OF EXPERIMENTS	CONTROL PERIOD (AVERAGES)			30–40 MIN AFTER GIVING DRUG		
		Vol.	Sp g.	Total N	Vol.	Sp g.	Total N
		cc		mgm./cc.	cc.		mgm /cc.
1-44	2	6.25	1.0111	2.15	4.8	1.0105	1.75
7-43	1	7.3	1.0106	1.8	4.5	1.0097	1.3
3-44	1	4.07	1.0110	2 1	4.2	1.0102	1.6

used to evoke secretion. This fact strongly supports the current view that the parasympathetic innervation is a factor in promoting the secretion of enzymes by the pancreas. (For literature see Thomas (22)). It does not necessarily follow that each effective stimulus initiates a parasympathetic reflex. Such an interpretation would be absurd in the case of secretin and is usually not accepted in the case of HCl (23 p 499). A more satisfactory explanation for the change in response to secretin, at least, is to be found in the well known "tonic" activity of the abdominal vagus, possibly augmented in specific instances by conditioned reflexes. Such activity, though generally subthreshold for pancreatic secretion, may nevertheless be capable of modifying the response to effective stimuli and thus increase the enzyme content and even the volume of the secretion. We see no reason, however, to deny the possibility of a reflex response to HCl in the intestine. The fact that the response to HCl is affected more by atropine than is the response to secretin favors the view that acid owes part of its action to a reflex. Further evidence is to be found in the observation by Crider and Thomas (18) that the volume of pancreatic juice secreted in response to HCl in the intestine is considerably reduced by section of the vagus nerves in unanesthetized dogs.

The results with soap are difficult to interpret. In the experiments of Crider and Thomas (18), the response of the pancreas to soap stimulation was not affected by cutting either the vagus or splanchnic nerves or both. Babkin (23 p 753) has suggested that a substance with choline-like properties, possibly choline itself, may be involved in stimulation of the pancreas by soap or fat in the intestine. Pancreozymin (24) apparently is not involved since the action of this agent is said to be unaffected by atropine.

The considerable decrease after atropine in specific gravity and total nitrogen of pancreatic juice secreted in response to peptone stimulation is in accord with the view expressed by Crider and Thomas (18) that peptone owes a part of its action on the pancreas to reflexes involving the vagus nerves. The increase in volume of secretion that occurred in most dogs under the same circumstances does not accord with the results of vagotomy. This operation was followed by a decrease in volume. The fact that the secretory response to peptone was abolished after atropine in those dogs in which the stimulus was applied to an isolated loop of the intestine eliminates the possibility that the increase in volume is due to local changes in the pancreas such as relaxation of the ducts or direct stimulation of the secreting cells. This fact also eliminates the possibility that the increase in volume results from increased effectiveness of a hormone from the intestinal mucosa. Evidently some mechanism in the intestine capable of promoting pancreatic secretion is stimulated by atropine and hyoscyamine. Stimulation of the enteric nerve plexus by these drugs would explain the results if it could be shown that the plexus extends into the pancreas and conveys secretory impulses.

SUMMARY

1. Doses of atropine or hyoscyamine sufficient to block the secretory endings of the vagus nerves decreased the specific gravity and total nitrogen of the pancreatic juice in unanesthetized dogs regardless of the stimulus used to promote secretion.

2. When the stimulus was soap or HCl in the intestine or intravenous secretin the volume of the secretion was also decreased by the action of these drugs. The response to secretin was affected least.

3. The volume of pancreatic juice obtained in response to peptone in the intestine was increased by atropine or hyoscyamine in seven of ten normal dogs.

4. The secretory response of the pancreas which normally follows injection of peptone into a modified Thiry loop of the duodenum or jejunum was abolished by atropine or hyoscyamine.

CONCLUSION

Either the parasympathetic innervation or some other mechanism that is affected by the parasympathetic depressants contributes to the secretion of enzymes by the pancreas in response to a variety of stimuli including HCl and secretin.

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STUDIES ON THE PHARMACOLOGY OF FLUOROACETATE

I. Species Response to Fluoroacetate

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Sodium fluoroacetate has recently been introduced as a rodenticide (1). Therefore, an understanding of its pharmacological actions and a knowledge of its toxicity to various species, including primates, is essential.

In a cooperative program with the Fish and Wildlife Service of the Department of Interior, the Medical Division of the Chemical Warfare Service in association with the Army Committee on Insect and Rodent Control has conducted studies on fluoroacetate. These have revealed numerous pharmacological actions, some of which may prove to be valuable as research tools. An extreme degree of species variability in both site of action and susceptibility has also been demonstrated.

The fluoroacetate ion might be expected to share the actions of other monohalogenated acetates (2, 3). This expectation has not been fulfilled. Rather, it is much more toxic and exerts specific actions on the myocardium and nervous system. Death in acute fluoroacetate poisoning results either from ventricular fibrillation or excessive stimulation of the central nervous system. The following paper deals with a general survey of the actions of fluoroacetate in a variety of mammalian species. Emphasis has been placed on species variability with respect to site of action and susceptibility. A more detailed analysis of the mechanism of action of fluoroacetate will be presented in further communications.

Procedure. There is a variety of compounds which yield the fluoroacetate ion in the body. These include fluoroethanol, methyl fluoroacetate and sodium fluoroacetate. All possess the same pharmacological actions when compared on the basis of equimolar dosage. However, inasmuch as sodium fluoroacetate may be contaminated with fluoride ion unless special precautions are taken in its preparation, in all the following experiments methyl fluoroacetate, which can readily be purified by distillation, was employed.

Methyl fluoroacetate is a colorless, moderately water-soluble (*ca.* 8 per cent) liquid with a fruity odor boiling at 104-105°C. (uncorr.). In aqueous solution it hydrolyzes with time to form fluoroacetic acid and methyl alcohol. This can largely be avoided by storing solutions in the refrigerator. On the other hand the carbon-fluorine bond can only be split by such harsh procedures as pyrolysis in a free flame or treatment with hot chromo-sulfuric acid.

The actions of fluoroacetate have been studied in a wide variety of mammalian species as well as chickens and frogs. For administration in all species the methyl fluoroacetate was dissolved in isotonic sodium chloride solution and injected by an appropriate route.

Results. *I. Species in which fluoroacetate affects chiefly the heart. A. Rabbit.* Following the intravenous injection of an LD_{90} of methyl fluoroacetate

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(0.5 mgm./kgm., see table 1) there is a latent period of one to two hours during which the animal appears normal. At this time a small proportion of animals may develop a sudden convulsive seizure of a tonic-clonic variety, terminating in opisthotonus, pupillary dilatation and death. No heart sounds are audible although gasping respiratory movements continue for several moments. If the heart is examined at this point the ventricles will be found to be fibrillating. Detailed electrocardiographic studies of the events preceding fibrillation will form the subject of a future communication.

Usually, however, rabbits develop motor disturbances about two hours after injection which are manifested by a sprawling position of the forelimbs with the head placed flat on its side between them. This phenomenon has not been analyzed further. Between two and twelve hours after injection ventricular fibrillation occurs and is immediately followed by an anoxic convulsion. The rabbit, in common with many small mammals, will often spontaneously revert to a normal sinus rhythm during a period of ventricular fibrillation. If the myocardium has not been anoxic for too long a period the animal may recover sufficiently to become ambulatory. However, a second anoxic convulsion due to the recurrence of ventricular fibrillation soon follows. Rarely does any animal repeat this performance more than twice, usually dying in the second bout of fibrillation.

TABLE 1

Mortality of rabbits following intravenous injection of methyl fluoroacetate

Data obtained over a two year period with several breeds of rabbits

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
0.10	10	0	0
0.15	10	0	0
0.20	10	1	10
0.25	16	9	56
0.50	101	94	94
1.0	53	53	100

In order to establish with certainty that recurrent convulsions were due to bouts of cardiac syncope, several unrestrained rabbits were poisoned with fluoroacetate and their electrocardiogram continually observed by means of the Sanborn "Cardioscope". In every instance circulatory standstill preceded the convulsions. Practically always this was due to ventricular fibrillation; occasionally A-V block or ventricular tachycardia was responsible. Further evidence that fluoroacetate fails to excite the nervous system of rabbits is the fact that electroencephalographic examination has failed to reveal the characteristic convulsant activity seen in species susceptible to the central actions of the fluoroacetates.

Except for the temporal aspects of the course of poisoning there is little difference in the response of rabbits to an LD_{50} (0.20 to 0.25 mgm./kgm.) and large doses (2.0 mgm./kgm.) of methyl fluoroacetate. In the case of the former dose,

death may be delayed for as long as 24 hours; in the case of the latter it may occur within 30 to 60 minutes.

The mortality data shown in tables 1 and 2 indicate an intravenous LD_{50} in the order of 0.20 to 0.25 mgm./kgm. These are combined data from several varieties of rabbits. There is some evidence that the small, short-eared breed of rabbits known as "Dutch" are less susceptible than other breeds. Therefore, the slightly lower toxicity following subcutaneous injection is probably more related to susceptibility than route of administration. As will be seen for the rat, toxicity by various routes is very similar.

B. Goats. Twenty-four goats were divided into six groups of four each and injected intramuscularly with methyl fluoroacetate at dose levels of 0.6, 0.7, 0.8, 0.9, 1.0, and 1.1 mgm./kgm., respectively. At levels of 0.7 and 0.9 mgm./kgm. all the animals died but in the remaining dose-groups only three of the four animals died. Most of the deaths occurred between four and twenty hours after injection although several animals died one or two days later and one was observed by chance to die with all the symptoms of ventricular fibrillation seven days after the injection. It is possible to state only that an intramuscular LD_{50} for the goat is somewhat less than 0.6 mgm./kgm.

TABLE 2

Mortality of rabbits ("Dutch") following subcutaneous injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
0.30	11	3	27
0.50	25	24	96

Four other animals were examined electrocardiographically, two under light pentobarbital anesthesia (35 mgm./kgm. by vein, frequently repeated) and two unanesthetized. Large doses of methyl fluoroacetate (2.0–4.0 mgm./kgm.) were given by vein and death occurred in from one to three hours. In all four animals death followed ventricular fibrillation. No return to sinus rhythm was noted, once ventricular fibrillation developed and hence no recurrent convulsions were observed. No evidence of any central nervous system action was obtained and in general the goat behaved as did the rabbit.

C. Horse. Two healthy over-age cavalry remounts were made available because of their vicious temper. Horse no. 1 received 0.5 mgm./kgm. of methyl fluoroacetate intravenously without marked effect upon his behavior in the corral during the remainder of the day. The following day the animal was depressed although still able to stand. No evidence of central nervous system stimulation was elicited. Three days later this horse was again normal and was anesthetized with 240 mgm./kgm. of sodium barbital by vein. Control electrocardiograms were obtained and 3.2 mgm./kgm. of methyl fluoroacetate were injected intramuscularly. Three hours later the peripheral pulse was impalpable and in another hour ventricular fibrillation ensued. There was no

change in the appearance of the animal until after ventricular fibrillation was detected electrocardiographically. It is difficult to be sure that no central nervous system changes occurred since the animal was anesthetized. However, in species susceptible to the central actions of fluoroacetate barbiturate anesthesia does not completely mask central excitation (*vide infra*). Horse no. 2 received 1.75 mgm./kgm. and developed the same picture. The response of the horse therefore closely resembles that of the goat and the rabbit. It would appear that the horse is somewhat more resistant than the goat for although the animals were not actually weighed the estimates of their weight made by several experienced veterinary officers did not vary more than fifty pounds from each other. Thus a lethal dose for the horse would appear to be between 0.5 mgm. and 1.75 mgm./kgm., intramuscularly, and to exert its action primarily upon the myocardium.

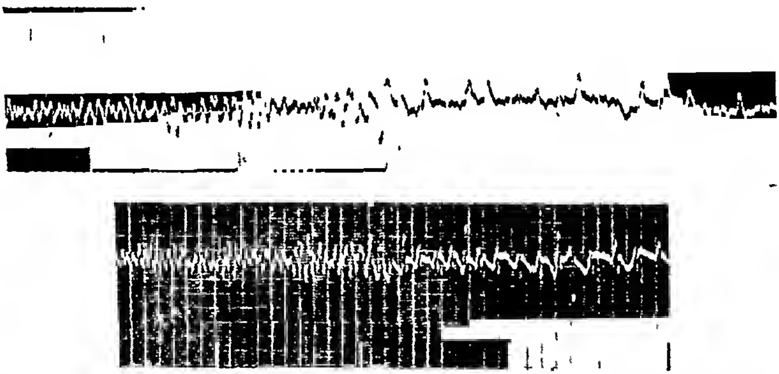

FIG 1. The upper electrocardiographic tracing (Lead II) from a Spider monkey reveals the onset of ventricular fibrillation six hours after the intravenous injection of 12.0 mgm./kgm. of [illegible] The lower record was obtained from a small pig three hours [illegible] the intraperitoneal injection of 0.8 mgm./kgm. of methyl [illegible] fibrillation was preceded by a run of premature ventricular contractions.

II. Species in which fluoroacetate affects heart and central nervous system. A. Cat. The response of the cat to fluoroacetate differs from that of the animals previously described in that both the heart and the central nervous system are involved. Intravenous doses of 0.5 to 1.0 mgm./kgm. of methyl fluoroacetate in unanesthetized cats, following the characteristic latent period, produced vomiting, salivation, pupillary dilatation, hyperpnea, hyperexcitability and myotonic convulsions occasionally interspersed with a violent myoclonic convulsion. This convulsive state persisted for several hours. In general, the cardiac changes in unanesthetized cats were those compatible with anoxia. Death in these animals was usually due to depression of the respiratory center while the heart continued to beat after respiration had ceased. Occasionally death resulted from ventricular fibrillation.

Cats anesthetized with sodium pentobarbital (35 mgm./kgm. intraperi-

toneally) and injected intravenously with 0.5 to 5.0 mgm./kgm. of methyl fluoroacetate manifested central nervous system stimulation even though completely anesthetized However, respiratory depression was not marked and only occasionally was artificial respiration required to tide the animal over an apneic period. Ventricular fibrillation occurred frequently in this group of animals.

Because of the uncertainty whether in a given cat cardiac or central nervous system symptoms would predominate, they were not frequently used for more detailed studies. For that reason little effort was made to obtain precise data on the toxicity of methyl fluoroacetate to cats. However, of 24 animals injected with 0 5 mgm /kgm or more, all died. Thus the LD_{50} is below 0.5 mgm./kgm. and probably in the neighborhood of 0.3 mgm /kgm.

B Domestic pig Small pigs weighing between two and ten kilograms were injected intraperitoneally with 0 5 mgm /kgm. of methyl fluoroacetate. One

TABLE 3

Mortality of small pigs following intraperitoneal injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm /kgm			
0 1	2	0	0
0 2	2	0	0
0 3	2	1	50
0 4	5	2	40
0 5	4	3	75
0 6	6	5	83
0 7	3	3	100
0 8	3	3	100
1 0	1	1	100

or two hours later occasional tremors and increased excitability were observed together with a disinclination to move in typical cases About four hours after the injection violent myotonic convulsions occurred which gradually regressed or ended in respiratory depression and death while the heart continued to beat vigorously for several minutes after respiration ceased. Approximately 50% of the animals died of respiratory failure The remainder were observed to develop ventricular fibrillation following or during convulsive seizures of central origin. An occasional animal died of ventricular fibrillation before central excitation was evident (see fig 1) Immediate post-mortem examinations usually revealed four or five cubic centimeters of bloody pericardial fluid These experiments were performed during the winter on animals kept in an only moderately warm sty and no unpoisoned animals were examined. It is possible that this change was not due to fluoroacetate, especially as it was not noted in any other species

As can be seen from the data presented in table 3, the LD_{50} of methyl fluoroacetate administered intraperitoneally to pigs lies close to 0 4 mgm./kgm. The action of the drug appears to be exerted more or less equally upon the

myocardium and the central nervous system with somewhat more than half the animals actually succumbing to ventricular fibrillation.

C. Monkeys. The monkey was chosen as the primary test animal in a search for an antidote to fluoroacetate. In view of the marked differences among the various species of animals studied, it was felt that the application of the results of studies on antidotes to man would be more feasible if data were available upon primates. Because of wartime procurement difficulties it was necessary to make as many therapeutic trails upon a given monkey as possible. For that reason animals served as "autocontrols", receiving the same dose of fluoroacetate several weeks after surviving a given dose with the aid of treatment. It was therefore

TABLE 4

Mortality of Macaca mulatta following the intravenous injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
5.0*	13	9	69
7.5	9	7	78
10.0	18	15	84
15.0	3	3	100

* Several large animals died within one hour or less following the injection of 5.0 mgm. In common with other experiences (7), it has been found that smaller and younger members of a species are more resistant to the induction of ventricular fibrillation by any means.

TABLE 5

The mortality of a species of spider monkey (Ateles geoffroyi?) following the intravenous injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
10.0	2	1	50
12.0	6	1	17
12.5	5	1	20
16.0	1	1	100
20.0	2	2	100

necessary in order to kill the animals when untreated to use doses of fluoroacetate well above the LD_{50}. This is reflected in the data in tables 4 and 5.

Two species of monkey were available for study, the common East Indian monkey, *Macaca mulatta* (Rhesus), and a prehensile-tailed South American spider monkey of the *Ateles* family, possibly *Ateles geoffroyi*. The majority of both species were examined unanesthetized, tied on their backs to an animal board. Approximately one third of the macaques were sedated with intravenous sodium pentobarbital (15–20 mgm./kgm.). The spider monkeys required no sedation. All injections were made into the popliteal veins of the macaques and the femoral veins of the spider monkeys.

1. *Macaca mulatta.* During the course of the studies on fluoroacetate, 43 of

these monkeys were injected with methyl fluoroacetate. Continuous cardioscopic observations of the electrocardiogram and continuous auscultation of the heart by means of a heart microphone, amplifier, and loud speaker permitted moment to moment assessment of the cardiac status.

A typical experiment is that performed on monkey no. 5. Ten milligrams per kgm. of methyl fluoroacetate were injected intravenously with the animal prepared as described above. One hour later occasional premature ventricular contractions were noted. At two hours the T-wave of the electrocardiogram had become accentuated. A few minutes later a convulsion was heralded by blinking, defecation, nystagmus, facial muscle twitching and some salivation. After what appeared to be strikingly like the onset of an epileptic attack a jerking, symmetrical tonic convulsion occurred. This convulsion lasted for three or four minutes and gradually subsided, the animal remaining conscious but manifestly fatigued. Shortly after this convulsion, the electrocardiograph revealed persistent alternation in the shape of the T-wave. Alternation was observed in the peripheral pulse and could be detected in the heart sounds. One hour later a second, very brief, convulsive episode occurred, a phenomenon which was not usually encountered. Four and one-half hours after injection, a marked alternation of the T-wave and a 50 per cent pulse deficit were persistent. About one half hour later ventricular fibrillation ensued and was followed by a typical anoxic convulsion. The injection of procaine (1.0 cc. 2% procaine hydrochloride) into the heart combined with transthoracic cardiac massage reverted the fibrillation to an organized contraction within one minute. Bouts of ventricular fibrillation recurred four more times in the next two hours and all but the final bout were terminated with procaine and cardiac massage.

It is evident that in the macaque death is due entirely to ventricular fibrillation although a typical convulsion of central origin is seen in nearly every individual. These convulsions can be ablated or averted by the intravenous administration of sedative doses of sodium pentobarbital (15–20 mgm./kgm.) and even if untreated are not sufficiently severe to cause death. It was apparent, therefore, that the search for an antidote should be directed toward prevention or reversal of ventricular fibrillation. The fact that the intracardiac injection of procaine and cardiac massage can restore an organized beat to a fibrillating heart has been demonstrated by Burstein and Marangoni (4). The efficacy of this procedure in terminating fibrillation induced by methyl fluoroacetate in the macaque was soon established. For example, one animal was reverted to sinus rhythm after ten successive bouts of ventricular fibrillation, each confirmed electrocardiographically. Death resulted in the eleventh episode. It became commonplace to detect ventricular fibrillation immediately by means of the heart microphone and to render treatment which was effective in approximately ninety per cent of the incidents. However, only two animals were permanently saved by this method, recurrent attacks of ventricular fibrillation eventually becoming irreversible.

In an analysis of the cardiac actions of procaine Marangoni, Burstein and Rovenstine (5) have ascribed to the para-amino benzoic acid portion of the

procaine molecule an action on the myocardium which is effective in preventing the onset of epinephrine induced ventricular fibrillation in dogs under cyclopropane anesthesia. For this reason the sodium salt of this acid was prepared and tested as an antidote in fluoroacetate poisoning. However, oral doses as as high as 300 mgm./kgm. administered hourly throughout the experiment failed to alter significantly the incidence of ventricular fibrillation in 21 monkeys, although a general impression was gained that PABA rendered defibrillation with procaine easier.

Two other drugs alleged to have actions on the myocardium effective in the prevention of ventricular fibrillation, namely papaverine (6) and quinidine (6) were examined. Papaverine hydrochloride (10 mgm./kgm., intravenously) was found to be of no benefit. In fact, such doses lowered QRS voltage and slowed cardiac rate markedly for several minutes after each injection. Likewise the prophylactic administration of quinidine (30.0–50.0 mgm./kgm. of the hydrochloride by mouth) failed completely to prevent ventricular fibrillation following 10 mgm./kgm. of methyl fluoroacetate, even when administered every three hours during the course of the poisoning. In four macaques treated in this manner and three others treated with intravenous quinidine (5.0–10.0 mgm./kgm. every three hours) there was no evidence of any protective action.

It is thus apparent that the only treatment to date which has been at all successful in reversing ventricular fibrillation has been the use of intracardiac procaine with cardiac massage. However, attempts to treat the spider monkeys were not nearly so successful possibly because this species has a much wider and heavier thoracic cage than the macaque. This rendered cardiac massage ineffective and indicates that massage may play an important role in the reversal to a normal contraction. The possible value of this procedure in man may therefore be questioned.

2. *Ateles* (*geoffroyi?*). These were large (4–6 kgm.) spider monkeys in relatively poor condition, several dying spontaneously in the animal house during the period of a month. The course of fluoroacetate poisoning in these animals is similar to that described for the macaque with two major differences. The first is the nearly complete absence of central nervous system manifestations. Only one monkey of the series of 16 developed a convulsion, which was of exactly the same type as described for the macaque. None of the remaining 15 exhibited the slightest sign of central nervous system excitation. The second is the increased resistance to fluoroacetate shown by the spider monkey. Tables 4 and 5 reveal that the spider monkey is at least two or three times more resistant to fluoroacetate. Inasmuch as death in both species may be ascribed entirely to the cardiac action of fluoroacetate, the fact that one species manifests central nervous system stimulation and one does not, may be regarded as an unimportant factor in the difference.

III. Species in which fluoroacetate affects only the central nervous system. A. Dog. Following the intravenous injection of as little as 0.10 mgm./kgm. of methyl fluoroacetate there is a latent period of one to two hours. During this period the animal is apparently normal. With larger doses the latent period is

somewhat, but not markedly, shortened. The onset of central nervous system stimulation is indicated by the rather sudden appearance of hyperexcitability, the animal running about and barking vigorously. Within a few minutes, this period of hyperexcitability gives way to frank convulsions. These are usually heralded by snapping and barking, some salivation, defecation, blepharospasm and twitching of the facial muscles. The convulsion itself is at first myotonic although later the action of the drug tends chiefly to be manifested by running movements, especially of the forelimbs, with an occasional brief tonic episode. Barking and panting persist during the clonic phase of the convulsive period. Nystagmus is occasionally observed, particularly at the end of a brief quiescent period, when it heralds the commencement of a severe convulsive episode. The convulsions may persist for one or two hours, ending in respiratory failure, the heart continuing to beat for some time after respiratory movements have ceased.

Anesthetized animals (sodium pentobarbital, 35 mgm./kgm. by vein) show evidence of the extreme central nervous system stimulation despite the anesthesia. No cardiac changes have been observed in either anesthetized or unanesthetized dogs which were not the direct result of the anoxia produced by respiratory failure. One animal was placed under artificial respiration when spontaneous breathing ceased after an injection of 1.0 mgm./kgm. of methyl fluoroacetate. Approximately one hour later ventricular fibrillation developed. It may be taken as axiomatic that the central nervous system alone reacts significantly to fluoroacetate in the intact dog. Although it is apparent that ventricular fibrillation can result from the action of the drug on the dog's heart under certain circumstances, under ordinary conditions death results from respiratory depression during the convulsions while the cardiac status is completely unaffected by the drug. A detailed analysis of the action of fluoroacetate on the central nervous system will appear in a later communication from this laboratory.

TABLE 6

Mortality of dogs following the intravenous injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
0.03	2	0	0
0.04	4	0	0
0.05	1	1	
0.10	7	6	86
0.20	4	4	100
0.30	3	3	100
0.45	3	3	100
0.50–2.0	7	7	100

It is apparent from examination of table 6 that the dog is the most susceptible species as yet examined. While not accurately established, the LD_{50} lies in the vicinity of 0.06 mgm./kgm. This dose is about twenty-five per cent of the

LD_{50} for the rabbit, the next most suceptible species and about one sixth of the LD_{50} for the guinea pig, the other species grouped with the dog.

B. Guinea pig. The sensitivity of the guinea pig approaches that of the rabbit rather than that of the rat and hamster. The LD_{50} following intraperitoneal injection approximates 0.35 mgm./kgm. (table 7). The behavior of these animals when poisoned with fluoroacetate is similar to that of the dog. Long continued convulsions are noted which alternate with a tremorous state. Convulsive bouts may be elicited by sounds or mechanical stimulation. Death always results from respiratory depression. No cardiac changes have been

TABLE 7

Mortality of guinea pigs following the intraperitoneal injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
0.10	3	0	0
0.20	5	0	0
0.30	6	1	16
0.35	14	7	50
0.40	5	4	80
0.50	3	3	100
0.75	3	3	100
1.00–5.00	31	31	100

TABLE 8

Morality of rats injected by various routes with methyl fluoroacetate

ROUTE AND DOSE	DIED/TOTAL	PER CENT MORTALITY
Per os (stomach tube)		
5.0 mgm./kgm. . ..	18/24	75
Intramuscular		
5 0 mgm./kgm. ..	13/25	52
Subcutaneous		
5.0 mgm./kgm.	6/12	50
6.0 mgm./kgm.	26/36	72

observed electrocardiographically which could not be explained by the increasing anoxia. None of the typical cardiac abnormalities observed in other species was noted.

IV. Species which react atypically to fluoroacetate. A. Rat. The symptoms of fluoroacetate poisoning in the rat are unlike those described for any of the other animals used in this study, with the exception of the hamster (q.v.). Following the usual latent period of one or two hours the animals are noted to be tremorous and hyperexcitable. Convulsions of the myotonic type can frequently be elicited by mechanical stimulation. Occasionally they are observed without apparent excitation. For the most part the rats tend to remain huddled together, heads curled under, and refuse food or water. Death when it occurs early (4–6 hours)

appears to be entirely due to respiratory depression. The following day the animals which have survived appear very depressed, and decidedly weak and ataxic. Examination of the heart at this time reveals a most extreme bradycardia. The pacemaker appears to be almost entirely ventricular and the rate may be as low as thirty contractions a minute. Several days may elapse before these animals return to normal. Ventricular fibrillation on the second day has been detected electrocardiographically, although infrequently.

B. Hamster. Small Syrian hamsters weighing from fifty to one hundred grams were injected intraperitoneally with methyl fluoroacetate. The behavior of this species was exactly as described above for the rat. As indicated by the data in table 9, the LD_{50} lies between 2.5 mgm. and 5.0 mgm./kgm.

V. Reaction of species other than mammals. A. Birds. Five Rhode Island Red chickens were injected intravenously (wing vein) with doses of 5.0, 10.0, 15.0 and 20.0 mgm. of methyl fluoroacetate. Death occurred in the two birds receiving 20.0 mgm./kgm. and in the one receiving 15.0 mgm./kgm. The other two birds survived having shown little or no symptoms of intoxication. Both the central nervous system and the heart were affected by fluoroacetate. All birds

TABLE 9

Mortality of hamsters following intraperitoneal injection of methyl fluoroacetate

DOSE	TOTAL ANIMALS INJECTED	TOTAL ANIMALS DYING	PER CENT MORTALITY
mgm./kgm.			
1.0	4	0	0
2.5	4	2	50
5.0	8	5	62
7.5	4	4	100

which died had persistent convulsions but death was due to ventricular fibrillation in the one case which was examined electrocardiographically.

B. Frogs. Very few frogs were examined as it was apparent from the first that they differed markedly from mammals in susceptibility. It was noted, however, that the LD_{50} lay in the vicinity of three hundred milligrams per kilogram. Furthermore, it was clear that the primary action of fluoroacetate was on the nervous system, no cardiac effects being noted. Twenty-four hours after poisoning the frogs were no longer convulsant but appeared to be paralyzed. In animals which recovered, this flaccid paralysis passed off during the subsequent twenty-four hours and the animals were apparently normal.

DISCUSSION. The character of the response of a number of species to fluoroacetate has been described. The various mammals fall into four definite groups when classification is based upon the degree of response of the heart and central nervous system to fluoroacetate (table 10).

The rabbit, goat, spider monkey and horse form the first group. These animals develop ventricular fibrillation without evidence of central nervous system stimulation. The cat, pig, and rhesus monkey form a second group in which both cardiac and central nervous system functions are affected, although not in

the same proportions in the three species. In the cat the central nervous system is primarily affected; in the pig there is a roughly equal partition between the action on the heart and the action on the central nervous system, while in the rhesus monkey the heart is primarily affected and the central nervous system stimulation is brief and unimportant. The third group, in which only central nervous system stimulation is observed, consists of the dog and the guinea pig. The rat and hamster show little relation to the previous three groups in their reaction to fluoroacetate and are, therefore, placed in a fourth group, that of atypical response.

TABLE 10

A summary of the responses of various mammals to fluoroacetate

The route of administration is intravenous unless otherwise specified

	LD_{50} MGM./KGM. OF METHYL FLUOROACETATE	CARDIAC RESPONSE	CNS RESPONSE
Group I			
Rabbit	0.20–0.25	Ventricular fibrillation	None
Goat	0.60	" "	"
Horse	0.50–1.75 (i.m.)	" "	"
Spider monkey	14.0	" "	"
Group II			
Cat	0.5	Slight	Marked
Pig	0.4 (i.p.)	Response of both heart and brain about equal	
Rhesus monkey	4.0	Ventricular fibrillation	Very slight
Group III			
Dog	0.06	None	Marked
Guinea pig	0.35 (i.p.)	"	"
Group IV			
Rat	5.0 (i.m., s.c.) 4.0 (per os)	Atypical	See text
Hamster	2.5–5.0 (i.p.)	"	" "

The wide range in lethality exhibited by fluoroacetate among the various species of mammals studied reveals about a two-hundred-fold difference between the most susceptible (dog) and the least susceptible species (spider monkey). Comparison of two species, both of which die in ventricular fibrillation (rabbit and spider monkey), reveals a nearly fifty-fold difference in susceptibility. As these mortality data were obtained by the intravenous injection of fluoroacetate in both species, which eliminates such factors as absorption, it is not unlikely that there is an underlying difference in the myocardium of these species capable of explaining a fifty-fold difference in the LD_{50}.

It is probable that the qualitative differences in response to fluoroacetate

among the various species can be explained by the uneven distribution throughout nature of the metabolic systems affected by fluoroacetate. Examination of table 10 reveals a distinct correlation between the degree of herbivorousness and the incidence of ventricular fibrillation as a cause of death and some correlation between carnivorousness and the occurrence of central nervous system stimulation. Undoubtedly, underlying this phenomenon are marked differences in the species distribution of the cellular systems disrupted by fluoroacetate.

The occurrence of a prolonged latent period between the injection of fluoroacetate and the development of symptoms of poisoning is of interest. It may be shortened by the employment of very large doses but under ordinary conditions neither the size of the dose nor the route of administration have any bearing upon the latent period. The route of administration does not significantly affect the toxicity of fluoroacetate. Although data are presented in detail only for the rat and rabbit, it has been observed frequently that various routes of administration produce death at the same dose level.

Taken in conjunction with the data in the body of the report, the considerations presented above indicate that fluoroacetate exerts a rather specific action upon one or more systems which are widely divergent in their distribution throughout the species studied. No evidence has been obtained that fluoroacetate is in any way comparable in its pharmacological action to the other mono-halogen derivatives of acetic acid.

CONCLUSIONS

1. The action of fluoroacetate injected as the methyl ester has been examined in eleven species of mammals and in the chicken and frog. No similarity to other mono-halogen derivatives of acetic acid has been noted.

2. The heart of the rabbit, goat, horse and spider monkey is primarily affected and death is due to ventricular fibrillation. No central nervous system action has been observed in these species, with the exception of one of 16 spider monkeys.

3. The central nervous system is primarily affected in the dog and guinea pig. Convulsions are epileptiform in character and death is due to the cessation of respiratory activity. No cardiac abnormalities have been observed.

4. The rat and hamster develop changes in which depression and delayed bradycardia are prominent. They do not usually exhibit ventricular fibrillation.

5. Both the heart and central nervous system are affected in the cat, pig, and rhesus monkey. The central nervous system action ultimately causes the death of a majority of cats, about half the pigs and none of the rhesus monkeys. Those individuals which do not succumb to respiratory failure during convulsions ultimately die of ventricular fibrillation.

6. The approximate LD_{50} in milligrams per kilogram of methyl fluoroacetate is as follows: dog 0.06, rabbit 0.20–0.25, guinea pig 0.35, pig. 0.4, cat 0.5, goat 0.5, horse 0.5–1.75, hamster 2.5–5.0, rhesus monkey (*Macaca mulatta*) 4.0, rat 5.0, spider monkey (*Ateles geoffroyi*) 14.0. Casual observation indicates that the chicken LD_{50} is of the order of fifteen milligrams per kilogram and the frog of the order of three hundred milligrams per kilogram.

ADDENDUM

Following completion of this manuscript a report by J. S. C. Marais in the *Onderstepoort Journal of Veterinary Science and Animal Industry* (**20**: 67–73 (1944), through *Chemical Abstracts*, **39**: 4116[5] (1945)) was received. This author found monofluoroacetic acid to be the toxic principle of *Dichapetalum cymosum*, a plant known to South African farmers as "Gifblaar". The lethal dose of the dried plant was found to be 1.0–1.5 gram/kgm. orally for rabbits, while the potassium salt of fluoroacetic acid was found to be lethal for rabbits in doses of 0.5 to 0.75 mgm./kgm. by any route. The author suggests that such simple fluorine substituted organic acids may be cheap and useful poisons and insecticides.

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THE RESPONSE OF THE ISOLATED FROG HEART TO DIFFERENT BARBITURATES

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Although Roth (1) noted the effects of certain barbiturates on the excised hearts of elasmobranchs, and Johnston (2) also studied the effects of some members of this group on the excised hearts of terrapin, no one seems to have made an extensive comparative study of the effects of the barbiturates on the excised hearts of frogs. To make such a study these experiments were undertaken.

METHOD. Large frogs of the species Rana pipiens were used. The animals were pithed and the heart exposed. By cutting a small opening into the left aorta, the tip of a modified Straub cannula was passed through the conus arteriosis (Bulbus cordis) into the ventricle. The cannula was securely fixed by a ligature around the aorta. The ventricle was then excised with the sinus venosus and auricles still attached to it and the whole suspended from an upright iron stand by means of the Straub cannula held in place by a clamp. The apex of the heart was connected to a light muscle lever. Changes in activity of the heart were recorded on the surface of a smoked drum. A chronographic marking key was placed directly beneath the tip of the writing lever.

The outer surface of the heart was kept moist by dropping Ringer's solution on it. This solution was the same as that used in perfusing the heart. The pressure within the ventricle was 2 cm. water pressure. Sodium salts of the barbiturates were dissolved in Ringer's solution which were made up fresh for each experiment. By means of a pipette the Straub cannula and ventricle could be emptied when desired and the control solution replaced by another of Ringer's-barbiturate solution and vice versa. After each test of the effect of a barbiturate solution had been made the heart was bathed and perfused with Ringer's solution and as soon as it had recovered a solution of higher or lower concentration of the same barbiturate or of a different barbiturate was tested on the same organ.

RESULTS. All of the barbiturates act qualitatively similarly but quantitatively differently upon the excised frog's heart. In figure 1 can be seen the relative effects of the sodium salts of amytal, butisol, vinbarbital, evipal and pentobarbital upon the same heart and in figure 2 the comparative effects of butisol, vinbarbital and seconal are shown on another heart. In these experiments 1/500 molar solutions were used. As these figures show the quantitative effects of butisol, vinbarbital and evipal are similar, however, the depressant effects of amytal and pentobarbital are more marked. With amytal not only the height of the contractions but the rate also was definitely influenced. Solutions of seconal (figure 2 at 3) and ortal usually caused complete stoppage of the heart. If the heart is not exposed for too long a time to these toxic concentrations complete recovery occurs.

In tables 1 and 2 are collected the results of 388 experiments in which different concentrations of the barbiturates were tested on 64 excised frog hearts. In

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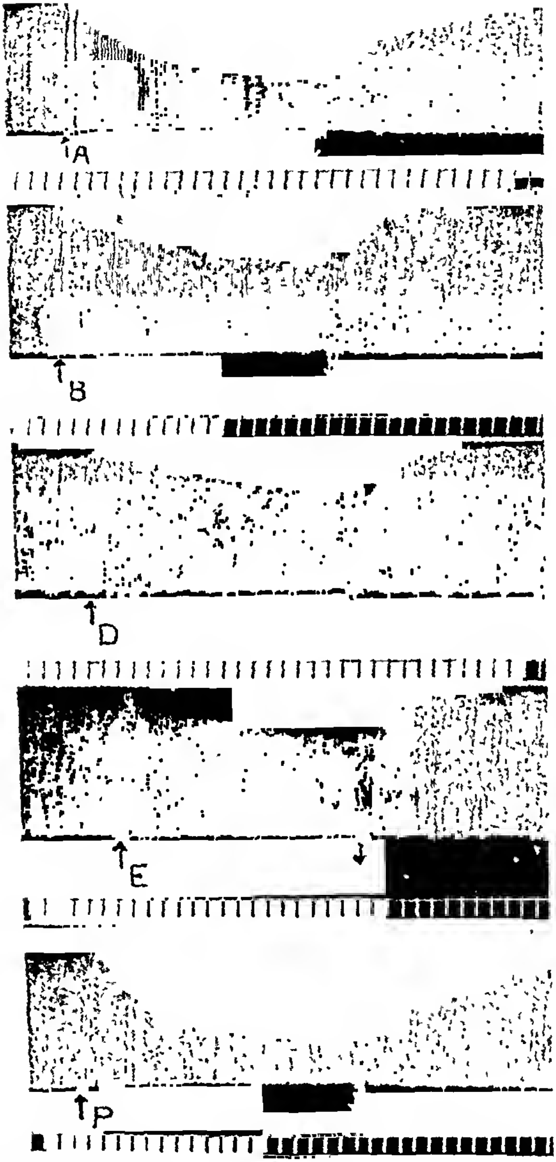

FIG. 1. The upper record in each curve is that of the contractions of the excised ventricle of the frog (Rana pipiens) and below it the time in intervals of 10 seconds. The upstroke of the lever indicates systolic contraction and the down stroke diastole. Between the arrows the Ringer's solution in the Straub cannula was replaced by M/500 solutions of the sodium salts of A, amytal, B, butisol, D, vinbarbital (delvinal), E, evipal, and P, pentobarbital.

table 1 the relative effects of the barbiturates on the height of the ventricular contraction are presented and in table 2 are the effects on the ventricular rate.

From these results it seems possible to divide the barbiturates studied into three groups, 1) those with marked cardiac depression; ortal and seconal, 2) those with moderate depression; amytal, pentobarbital and neonal and 3)

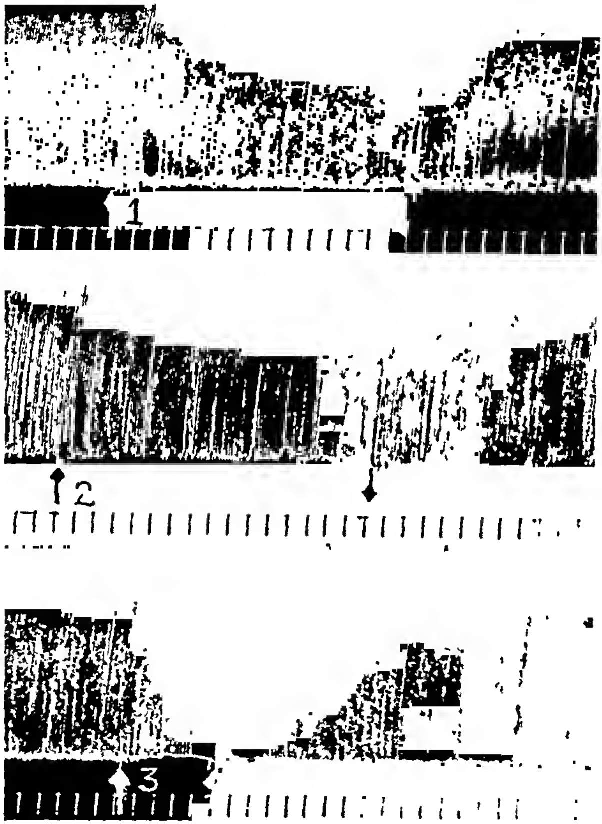

FIG. 2. Excised frog heart. M/500 solutions of the sodium salts of butisol, vinbarbital (delvinal) and seconal were substituted for the Ringer's solution in the Straub cannula between the arrows at 1, 2 and 3 respectively.

those with mild toxic effects; butisol, phenobarbital, evipal, vinobarbital and barbital.

It will also be observed in comparing the results in tables 1 and 2 that the rate is usually, though not always, slowed simultaneously with the decrease in the height of the contractions.

TABLE 1

Effects of the sodium salts of the barbiturates upon the height of contraction of the excised frog's ventricle

Three-hundred-eighty-eight experiments were performed upon 64 hearts. The barbiturates are arranged in a decreasing order of their efficiency. − indicated no effect; −+ the height of contractions decreased by less than 50 per cent; +− the height of contractions decreased by 50 per cent or more; + complete stoppage of the heart.

	M/3000			M/2000				M/1000				M/500				M/250				NUMBER OF EXPERIMENTS
	−+	+−	+	−	−+	+−	+	−	−+	+−	+	−	−+	+−	+	−	−+	+−	+	
Ortal	1	4	1			1	9			1	3				7					27
Seconal				1	4	4	5			4	11				9					38
Amytal				3	9				2	6	3			5	4				8	40
Pentobarbital					4			1	10	14	5		2	8	4				7	55
Neonal				2	1	1		3	3	1		1	5	3	2		3	2	2	29
Butisol				6	1			16	10			8	21	2	4		5	5	4	82
Phenobarbital								2	3			4	6	2			6	3	1	27
Evipal					3							3	7			1	7	1	2	24
Vinbarbital (Delvinal)				4	1			6	5			4	15	1	1	1	9	3	1	51
Barbital								4				5				3	3			15

TABLE 2

Effects of the sodium salts of the barbiturates upon the rate of contraction of the isolated frog heart

Sixty-four hearts were used for 388 experiments. The barbiturates are arranged in decreasing order of their efficiency in slowing the heart. − no effect; −+ heart slowed by less than 50 per cent; +− slowing of the heart was 50 per cent or more; + stoppage of the heart.

	M/3000			M/2000				M/1000				M/500				M/250				NUMBER OF EXPERIMENTS
	−+	+−	+	−	−+	+−	+	−	−+	+−	+	−	−+	+−	+	−	−+	+−	+	
Ortal	2	3	1			1	9			1	3				7					27
Seconal				1	4	4	5			4	11				9					38
Amytal				2	8	2		1	3	4	3		1	4	4				8	40
Pentobarbital				1	3			1	10	14	5		1	9	4				7	55
Neonal				3			1	2	4	1			6	3	2		2	3	2	29
Butisol				7				18	8			5	23	3	4		6	4	4	82
Phenobarbital								3	2			4	4	4			6	3	1	27
Evipal				2	1							2	7	1			8	2	1	24
Vinbarbital (Delvinal)				4	1			7	4			4	16		1	1	10	2	1	51
Barbital								4				4	1			5	1			15

SUMMARY AND CONCLUSIONS

1. All of the barbiturates studied depress the activity of the excised frog heart but they vary in the degree of this effect.

2. A given concentration of one barbiturate such as ortal sodium or seconal sodium may cause complete stoppage of the heart whereas the same concentration of another barbiturate such as barbital, vinbarbital, evipal, butisol or phenobarbital may produce no niticeable change in either the rate or the height of contractions.

3. Barbiturates can be grouped according to their toxic actions on the heart muscle.

The barbiturates used in this investigation were generously supplied to us by the following firms: Parke Davis and Company, Ortal sodium; Eli Lilly and Company, Seconal sodium, Amytal sodium; Abbott Laboratories, Neonal; Winthrop Chemical Company Inc., Evipal sodium; Sharp and Dohme Co., Delvinal sodium; and McNeil Chemical Company, Butisol sodium and Pentobarbital sodium.

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THE INHIBITION OF CHOLINESTERASE BY AROMATIC AMINO ALCOHOLS OF THE TYPE Ar-$CHOHCH_2NR_2$[1]

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During a search for compounds possessing antimalarial activity, Dr. Mosettig and coworkers prepared a series of aromatic amino alcohols (1) which proved to be effective antimalarials (2) with varying degrees of toxicity (11). Two of these compounds were investigated pharmacologically (unpublished experiments) and found to be hypotensive, to potentiate the action of nicotine and to have a pilomotor effect in dogs. Clinically one of these drugs caused a bradycardia, pilomotor activity and inability to relax the sphincter muscles controlling micturition (2). All of these autonomic effects pointed to a possible cholinergic action by the amino alcohols and consequently the compounds were tested for their effect on the activity of cholinesterase. Some of them were found to be extremely potent inhibitors of plasma cholinesterase, comparable to prostigmine, but much weaker as inhibitors of the esterase in red blood cells. The relationship between chemical structure and this inhibitory activity was investigated and an attempt made to correlate the degree of inhibition with toxicity and antimalarial activity.

METHOD. The inhibition of cholinesterase was determined by measurements of the rate of hydrolysis of acetylcholine using the method introduced by Ammon (3). This method depends on the reaction of the liberated acetic acid with bicarbonate, resulting in an evolution of CO_2 which was measured manometrically, using Barcroft differential manometers. The acetylcholine iodide (25 mgm.) was dissolved in 5 ml. of a bicarbonate buffered physiological salt solution (4) and 0.5 ml. delivered into the sidearm of the reaction flask. The plasma or laked red blood cells were placed in the main compartment of the flask, the drug added and the total volume of fluid brought to 3 ml. by addition of the bicarbonate solution. The manometers were then placed in the water-bath at 37°C. and flushed out with a mixture of 95 per cent N_2 and 5 per cent CO_2 previous to closing the stopcocks. After a period of 5 minutes to assure equilibrium the first reading was made and the contents of the sidearm were tipped into the main compartment. The second reading was made five minutes after tipping and subsequent readings were made at ten minute intervals. The initial rate was taken as the average rate (µl/min.) during the first ten minute interval.

Human blood plasma and red blood cells were used. The blood (approximately 25 ml.) was drawn from the cubital vein into a flask containing 0.4 ml. of 30 per cent sodium citrate and centrifuged at once. The red cells were washed four times with 0.9 per cent NaCl and then brought to a volume of 50 ml. with distilled water. Both the plasma and the hemolyzed cells were stored at 2°C. at which temperature they retained full cholinesterase activity for at least four months. The amount of red cells or plasma added to the flask varied between 0.7 ml. and 0.9 ml. depending on the activity of the sample of blood drawn. The plasma was diluted to five per cent and the red cells to one per cent before addition to the manometer flasks. The enzyme from the two sources showed almost identical activity at these dilutions under the experimental conditions outlined.

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[1] The work described in this paper was done under a transfer of funds, recommended by the Committee on Medical Research, from the Office of Scientific Research and Development to the National Institute of Health.

The drugs were furnished as hydrochloride salts by Dr. E. Mosettig and Dr. E. L. May of the National Institute of Health. With few exceptions the chemist's analytical samples were used to insure the purity of the preparation. Because the compounds, with the exception of the dimethylamino, diethylamino and dipropylamino alcohols, were relatively insoluble in water it was necessary to make all the initial dilutions in ethyl alcohol. The final dilutions were made with water so that the concentration of alcohol was 20 per cent or less. The concentration of the drug was so adjusted that approximately 0.1 ml. of the final dilution was added to the manometer flask. Since alcohol itself has a slight inhibiting

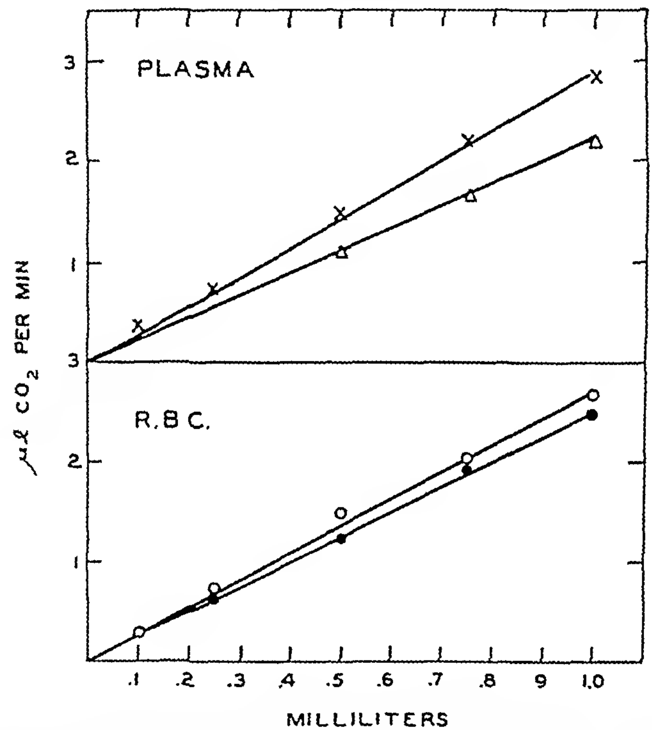

FIG. 1. THE RATE OF HYDROLYSIS OF ACETYLCHOLINE (0.003M) AT DIFFERENT CONCENTRATIONS OF HUMAN PLASMA OR HEMOLYEZD RED BLOOD CELLS
Abscissae represent milliliters of five per cent plasma or one per cent red blood cells (R.B.C.)

effect on cholinesterase, a control was run with each set of determinations containing the same amount of alcohol as was added with the drug. The inhibition by the drug was calculated as a per cent of this control value.

RESULTS. It was necessary to keep the concentration of acetylcholine rather low to avoid the inhibition due to excess substrate (5) when red blood cells were used as the source of enzyme. Having established that a concentration of 0.003M acetylcholine was optimal for the red cell enzyme it was then necessary to show that the hydrolysis was directly proportional to the amount of enzyme at this concentration. Figure 1 shows that there is such a relationship for both cells and

plasma. Each new sample of blood was tested as indicated in figure 1 but the data plotted represent only the samples that showed the widest variation in activity.

Table 1 shows the percentage inhibition of activity of human plasma cholinesterase in the presence of the amino alcohols indicated in the first column. The second column gives the drug number assigned by the Malarial Survey of the National Research Council and the third column the average rate of hydrolysis as μl of CO_2 per min. in the absence of drug. The next seven columns give the average percentage inhibition found at the concentration of drug indicated at the head of each column. The next to the last column gives the dose of the drug which, administered orally twice daily to eight doses, just allows one week old chicks to maintain their own weight. The data for this column were obtained from Dr. N. B. Eddy of this laboratory. The last column gives the dose of the drug which reduces parasitemia in the chick to twenty five per cent or less that of the controls. These data were obtained from Dr. G. Robert Coatney and Dr. W. Clark Cooper of this laboratory.

The first nine compounds of Table 1 constitute a series of phenanthrene-9-amino alcohols with the progressively increasing dialkyl amino group $-N(C_nH_{2n+1})_2$. The data for these compounds are plotted in figure 2A as per cent inhibition against drug concentration. The degree of inhibition increases from the dimethylamino to the dipropylamino derivative and then decreases rapidly. The dioctylamino and dinonylamino alcohols do not inhibit the enzyme at the concentrations tested. Plotting the per cent inhibition at 5×10^{-7} molar concentration of the drug against the number of alkyl carbons (fig. 3A) shows clearly that the inhibition passes through a maximum with propyl substitution.

In the analogous series of the tetrahydrophenanthrene-9-amino alcohols, comprising ten members (table 1), the inhibition again increases from the dimethylamino to the dipropylamino derivative and then decreases to zero with the higher members of the series (figure 2B). Figure 3A shows this progression when the inhibition at 5×10^{-7} molar concentration is plotted against the number of carbons in the alkyl group. The course of this curve is the same as was found for the non-hydrogenated phenanthrene derivatives but individually the tetrahydrophenanthrene derivatives are less active inhibitors than the corresponding phenanthrene analogs.

Replacing the diamylamino group in #1796 with a di-isoamylamino group (#3956) results in an increase in inhibition (figure 4). This change in inhibition is in the same direction, though not so pronounced, as is effected by the replacement of the diamylamino group by the dibutylamino group (#1795). In both cases the carbon chains of the alkyl group have been shortened. Conversion of the tertiary di-iso-amylamino group (#3956) to the secondary isoamylamino group (#5921) produced a further increase in the inhibition of the enzyme.

That the hydroxyl group on the side chain contributes to the activity of the compounds as inhibitors of plasma cholinesterase can be seen by comparing the results obtained with compound #1796 with #11580, #8845 and #2673 (table

TABLE 1

The percentage inhibition of the cholinesterase in human plasma by phenanthrene and tetrahydrophenanthrene amino alcohols

COMPOUND	SN‡	CONTROL	DRUG CONC. × 10^{-7} M: 22.4	11.2	8.98	5.6	4.48	2.24	1.12	TOL. DOSE*	THER. DOSE†
			Inhibition								
		μl./min.	%	%	%	%	%	%	%	m. mols kgm.	m. mols kgm.
p-9-$CHOHCH_2N(CH_3)_2$	1776	1.95	78	62		45		26			0.17
p-9-$CHOHCH_2N(C_2H_5)_2$	1777	1.95	93		80		70	45	23	0.34	0.14
p-9-$CHOHCH_2N(C_3H_7)_2$	1778	2.02	91	87	87		72	50	28	0.84	0.21
p-9-$CHOHCH_2N(C_4H_9)_2$	1779	2.03	88	85	82		64	41	26	0.65	0.13
p-9-$CHOHCH_2N(C_5H_{11})_2$	5242	2.01	64	42		27		12		0.73	0.15
p-9-$CHOHCH_2N(C_6H_{13})_2$	5480	2.02	23	13		5		0		1.36	0.14
p-9-$CHOHCH_2N(C_7H_{15})_2$	6827	2.01	10							1.06	0.034
p-9-$CHOHCH_2N(C_8H_{17})_2$	6828	1.98	2							1.4	0.064
p-9-$CHOHCH_2N(C_9H_{19})_2$	8867	1.88	0							1.9	0.060
-6-Cl p-9-$CHOHCH_2N(C_7H_{15})_2$	9160	1.84	1	0						1.2	0.031
p-9-$CH_2CHOHCH_2H(C_4H_9)_2$	13666	1.83	89	91		86		74	60	0.5	
p-9-$CH_2CHOHCH_2N(C_5H_{11})_2$	13669	1.88	87	88		69		46	30	0.35	
thp-9-$CHOHCH_2N(CH_3)_2$	1792	1.95	61	46		28		13		0.49	0.49
thp-9-$CHOHCH_2N(C_2H_5)_2$	1793	2.08	84	79		61		28		0.15	0.12
thp-9-$CHOHCH_2N(C_3H_7)_2$	1794	2.03	84	78		58		33		0.43	0.14
thp-9-$CHOHCH_2N(C_4H_9)_2$	1795	1.98	77	65		46		24		0.77	0.26
thp-9-$CHOHCH_2N(C_5H_{11})_2$	1796	1.86	40	25		14				1.2	0.15
thp-9-$CHOHCH_2N(C_6H_{13})_2$	5478	2.08	15	10						0.89	0.11
thp-9-$CHOHCH_2N(C_7H_{15})_2$	3957	2.08	6	0						1.05	0.07
thp-9-$CHOHCH_2N(C_8H_{17})_2$	3516	1.89	0	0						0.99	0.06
thp-9-$CHOHCH_2N(C_9H_{19})_2$	5241	1.80	5	0						1.9	0.12
thp-9-$CHOHCH_2N(C_{10}H_{21})_2$	5866	1.77	1	0						1.8	
thp-9-$CHOHCH_2N(i\text{-}C_5H_{11})_2$	3956	2.00	61	45		30		11		0.72	0.15
thp-9-$CHOHCH_2NH(i\text{-}C_5H_{11})$	5921	1.86	82	73		59	58	32		0.35	0.14
thp-9-$CHClCH_2N(C_5H_{11})_2$	8845	2.11	21	12		7				0.57	0.14
thp-9-$CH_2CH_2N(C_5H_{11})_2$	11580	1.94	31	15	13	6		4		0.11	
thp-9-$COCH_2N(C_5H_{11})_2$	2673	1.80	25	11						0.48	
thp-9-$CH_2CHOHCH_2N(C_2H_5)_2$	12984	1.97	89	89		79		52		0.29	
p-3-$CHOHCH_2NH_2$	10909	1.74	8	1		3				0.55	
p-3-$CHOHCH_2N(CH_3)_2$	1827§	2.13	27	15		6		8			
p-3-$CHOHCH_2N(C_2H_5)_2$	1764	2.10	71	55		30		12		0.60	0.76
p-3-$CHOHCH_2N(C_3H_7)_2$	1766	2.06	90	82		72	67	47	33	1.40	0.56
p-3-$CHOHCH_2N(C_4H_9)_2$	1767	1.84	85	63		52		28		1.30	0.52
p-3-$CHOHCH_2N(C_5H_{11})_2$	1768	1.90	41	27		14				0.72	0.36
p-3-$CHOHCH_2N(C_2H_4OH)_2$	1765	1.86	25	16		11				1.10	
-6-Cl p-3-$CHOHCH_2N(C_5H_{11})_2$	8978	1.96	23	15		12				1.10	0.06
-6-OH p-3-$CHOHCH_2N(C_2H_5)_2$	2662	1.83	24	15		6				0.72	

TABLE 1—*Continued*

COMPOUND	SN‡	CONTROL	DRUG CONC $\times 10^{-7}$ M 22.4	11.2	8.98	5.6	4.48	2.24	1.12	TOL. DOSE*	THER. DOSE†
			Inhibition								
		μl/min.	%	%	%	%	%	%	%	m.mols/kgm.	m.mols/kgm
p-2-$CHOHCH_2N(C_2H_5)_2$	1826§	2.00	68	54		31		12			
p-2-$CHOHCH_2N(C_3H_7)_2$	2666	1.96	76	67		46		31		0.56	0.56
p-2-$CHOHCH_2N(C_4H_9)_2$	2668	1.92	53	34		22				0.65	0.33
p-2-$CHOHCH_2N(C_5H_{11})_2$	5937	1.95	29	16						0.92	0.72
9,10-H_2P-2-$CHOHCH_2N(C_2H_5)_2$	1786	1.95	52	36		24				0.45	
-4-Cl Naphtha-1-$CHOHCH_2N(C_4H_9)_2$	5243	1.97	48	32		23				0.81	0.17
Naphtha-1-$CHOHCH_2CH_2N(C_4H_9)_2$	6816	1.96	74	66		48		25		0.29	

* Tolerated dose for chicks (see text).

† Therapeutic dose for chicks (see text).

‡ SN represents the identification number assigned to the drugs by the Malaria Survey Office of the National Research Council.

§ Numbers assigned in this laboratory.

p and thp represent phenanthrene and tetrahydrophenanthrene respectively.

1, figure 4). Reduction of the alcoholic group to the methylene group, oxidation to the keto group or replacement of the hydroxyl by chlorine all decreased the inhibitory activity.

Compounds of the general formula p-$CH_2CHOHCH_2NR_2$ are more active inhibitors of the plasma esterase than those of the formula p-$CHOHCH_2NR_2$. This is evident from a comparison of compounds #13666, #13669 and #12984 with their respective analogs #1779, #5242 and #1793 (table 1). Compound #13666 is in fact the most potent inhibitor of the phenanthrene derivatives tested, inactivating 50 per cent of the enzyme at a concentration of 0.6×10^{-7} molar.

When amino alcohol side chain is on the 3 position of the phenanthrene nucleus the compound is still an active inhibitor of the plasma cholinesterase but less so than the analogous 9 substituted compound. This is shown by comparison of compounds #10909 through #1768 with compounds #1776 through #5242 (table 1). The inhibition again passes through a maximum with propyl substitution (figures 2C and 3B).

Conversion of the diethylamino group of compound #1764 to a diethanolamino group as in #1765 reduces the inhibition more than one half. A similar reduction is affected by replacing the 6-carbon hydrogen of the nucleus with an hydroxyl group (#1764 and #2662). Replacing the same hydrogen with chlorine also reduces the inhibition as is shown by comparison of #1768 with # 8978.

Table 1 also contains data obtained on the inhibition of plasma cholinesterase by phenanthrene-2-amino alcohols (#1826 through #5937). While these compounds are less active inhibitors of the enzyme than the analogous compounds of the phenanthrene-3 or phenanthrene-9 series (figure 2D) they do show the same change in inhibitory activity with increasing length of the alkyl groups, passing through a maximum with the propylamino compound (figure 3B). Comparison

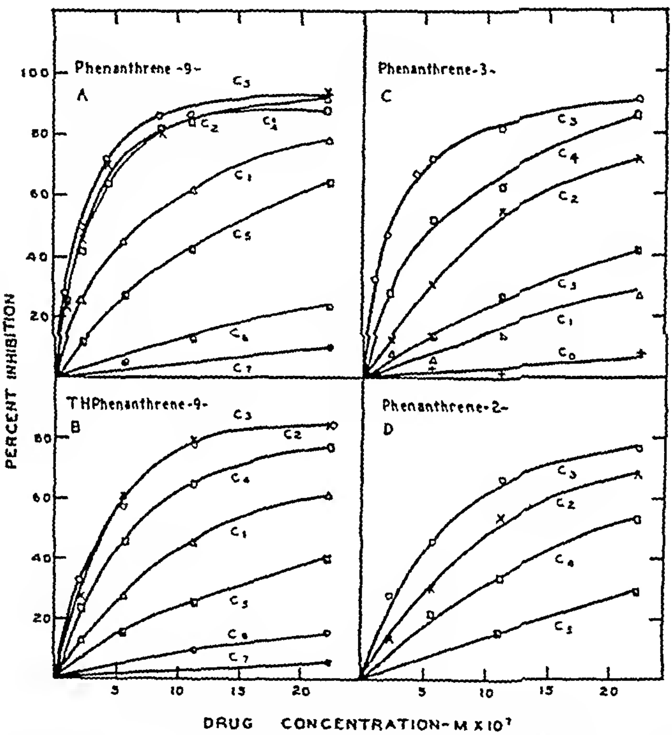

FIG. 2. THE PER CENT INHIBITION OF HUMAN PLASMA CHOLINESTERASE BY PHENANTHRENE AMINO ALCOHOLS

The number of carbons of the alkyl group on the nitrogen is indicated for each curve

of the results obtained with drug #1826 and #1786 shows that removal of the 9, 10 double bond by hydrogenation reduces the inhibition.

The last two compounds of table 1 are naphthalene derivatives. The data for these compounds show that the phenanthrene nucleus is not essential for the inhibition of the enzyme. Since the substituents on the naphthalene nucleus are not the same as any on the phenanthrene nucleus a direct comparison of their potency as inhibitors is not possible.

The phenanthrene and tetrahydrophenanthrene amino alcohols also inhibit the cholinesterase of human red cells (table 2) but the concentrations required

are of the order of 10^{-5} molar as compared with 10^{-7} molar for the plasma enzyme. Inspection of the data of table 2 as plotted in figure 5 shows that the inhibition by the propyl-, butyl- and amylamino derivatives reaches a limiting value with increasing concentration. This is due to the low solubility of these compounds in the bicarbonate solution. The point at which the curves break sharply approximates the maximum solubility of the compound. This factor of solubility limits the comparison of the potency of the compounds to a concentration below the maximum solubility of the least soluble member of the series. Comparison was consequently made at 1×10^{-5} molar (figure 6). At this concentration the tetra-

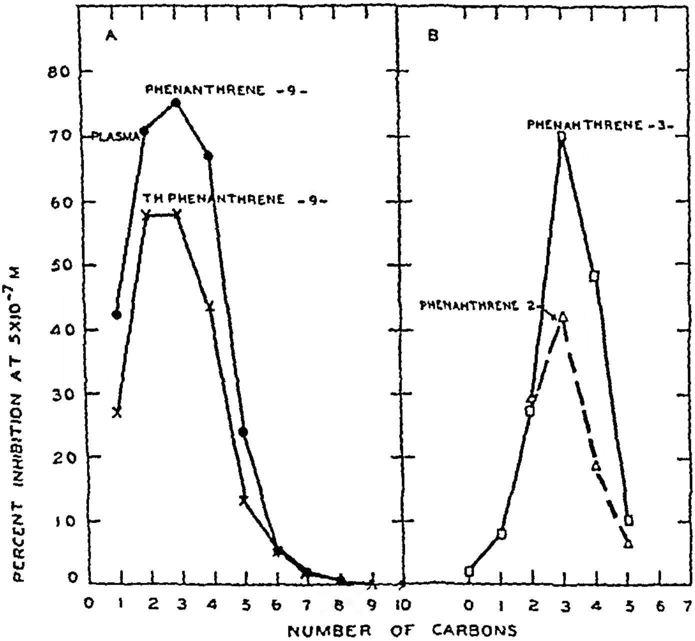

FIG. 3 THE VARIATION IN THE INHIBITION OF HUMAN PLASMA CHOLINESTERASE BY PHENANTHRENE AMINO ALCOHOLS AS A FUNCTION OF THE NUMBER OF CARBONS OF THE ALKYL GROUP ON THE NITROGEN

hydrophenanthrene derivatives are more active inhibitors than the phenanthrene derivatives, in contrast with the results obtained with the enzyme in plasma. Furthermore the inhibition increases with the size of the dialkylamino group up to the diamylamino compound. It was not possible to make measurements with the higher homologs because of their insolubility.

At the lower concentrations, where the factor of solubility does not interfere, the isoamyl derivative of tetrahydrophenanthrene (#5921) is a less effective inhibitor of the red cell enzyme than the amyl derivative (#1796). The propanolamino compound (#12984) is a less effective inhibitor of the red cell enzyme than the ethanolamino compound (#1793). With both these pairs the change in

effectiveness is in the opposite direction to that found when the plasma enzyme was used.

Drug #1796 is effective in the therapy of clinical malaria (2). Blood[2] was taken from 17 of the patients, before and during the administration of the drug, and tested for cholinesterase activity. In all but two cases there was a definite decrease in the enzymic activity after the administration of the drug. Typical results obtained, using 0.025 ml. of defibrinated whole blood, are plotted in figure

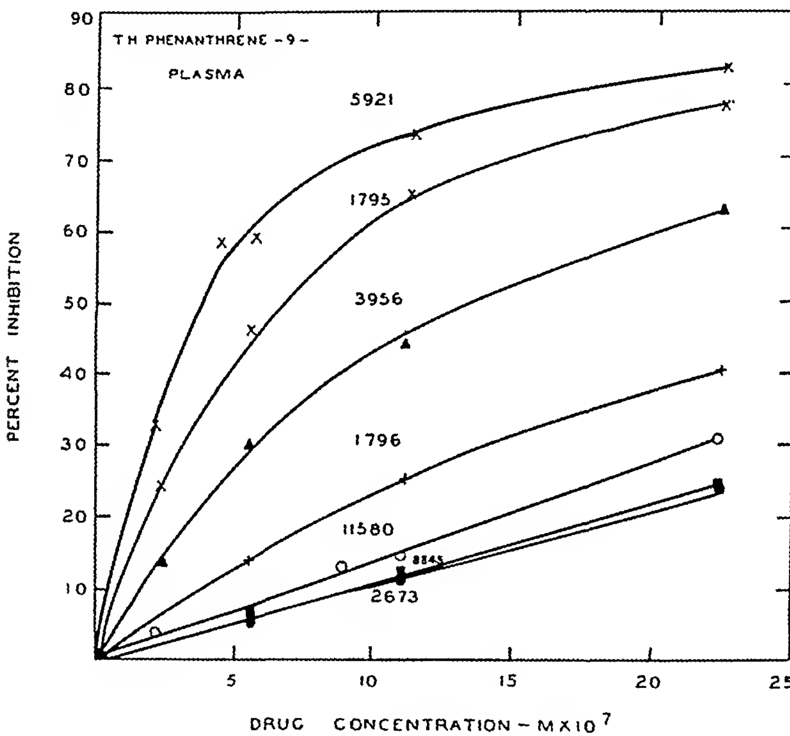

FIG. 4. THE VARIATION IN INHIBITION OF HUMAN PLASMA CHOLINESTERASE WITH CHEMICAL STRUCTURE

The number on each curve is the drug number given in table 1. For explanation see text.

7. All of the patients received 0.32 grams of #1796 three times per day for eight to ten days as indicated by the arrows on the graph. This dosage schedule was repeated on patients Yo and Se starting on the forty-third and thirty-ninth day respectively. Patient Dr received 0.1 gram of atabrine three times a day for seven days starting on the forty-second day. Patient Ma received no further medication after the initial treatment with #1796. The cholinesterase activity

[2] Thanks are extended to Miss Marie Gilson, Medical Technician, for drawing the blood for these experiments.

of the blood from all four patients fell to approximately two thirds of the premedication value during the first administration of the drug. The return toward normal was slow after withdrawing the drug, reaching the normal value after fifteen days in only one of the patients (Dr). The data presented in figure 7 suffer from lack of adequate controls since the effect of the malaria itself on the activity of blood cholinesterase is not known.

DISCUSSION. The original stimulus for this investigation was the possibility that a positive correlation might exist between cholinesterase inhibition and the antimalarial action of drugs, a possibility also considered by Waelsch and Nachmansohn (6). This would have led to a method of screening compounds for

TABLE 2

The percentage inhibition of the cholinesterase in human red blood cells by phenanthrene and tetrahydrophenanthrene amino alcohols

COMPOUND	SN*	CONTROL	DRUG CONC. × 10^{-5}M													
			64	48	45	32	22	16	12	8	5.6	4	2	1	.75	.5
			INHIBITION													
		µl/min.	%	%	%	%	%	%	%	%	%	%	%	%	%	%
p-9-$CHOHCH_2N(CH_3)_2$	1776	1.92	34	29		20		16		5						
p-9-$CHOHCH_2N(C_2H_5)_2$	1777	1.98	44	39		28		19		7						
p-9-$CHOHCH_2N(C_3H_7)_2$	1778	1.96	42	43		40		34		26						
p-9-$CHOHCH_2N(C_4H_9)_2$	1779	1.93						33	34	33		24	12			
p-9-$CHOHCH_2N(C_5H_{11})_2$	5242	1.92							22	23		22	20	18	11	6
thp-9-$CHOHCH_2N(CH_3)_2$	1792	1 89	50	44		30		15		6						
thp-9-$CHOHCH_2N(C_2H_5)_2$	1793	1.93	89		84	78	63	52	34	28	15					
thp-9-$CHOHCH_2N(C_3H_7)_2$	1794	1.88	72	72		72		72		57		36	22			
thp-9-$CHOHCH_2N(C_4H_9)_2$	1795	1.97						37	43			39	36	21		7
thp-9-$CHOHCH_2N(C_5H_{11})_2$	1796	1.95										23	27	27	19	10
thp-9-$CHOHCH_2NH(i\text{-}C_5H_{11})$	5921	1.81							69	63		39	25	13		
thp-9-$CH_2CHOHCH_2N(C_2H_5)_2$	12984	1.89	80	74		58		35		20						

* SN represents the identification number assigned to the drugs by the Malaria Survey Office of the National Research Council.

p and thp represent phenanthrene and tetrahydrophenanthrene respectively.

therapeutical testing. There is no such relationship between the inhibition of human plasma cholinesterase and antimalarial activity in the chick (table 1) and probably not in man since drugs #1796 and #5241 are equally effective antimalarials as judged by the oral dose required to suppress parasitemia (2). Since the inhibition of the human red cell enzyme increases with the size of the dialkylamino group up to the amylamino compounds (table 2, figure 6) without a corresponding increase in therapeutic acitivity, it can also be concluded that the inhibition of the enzyme from this source is not correlated with the antimalarial effect.

A casual examination of table 1 might lead to the conclusion that a positive correlation exists between the inhibition of the plasma enzyme and the toxicity of the amino alcohold listed there. However, a closer examination of pairs of com-

pounds, chosen to avoid complicating factors such as solubility, readily shows that no prediction can be made as to the tolerated dose in the chick from the data on the inhibition of the enzyme. The data do not preclude the possibility of a

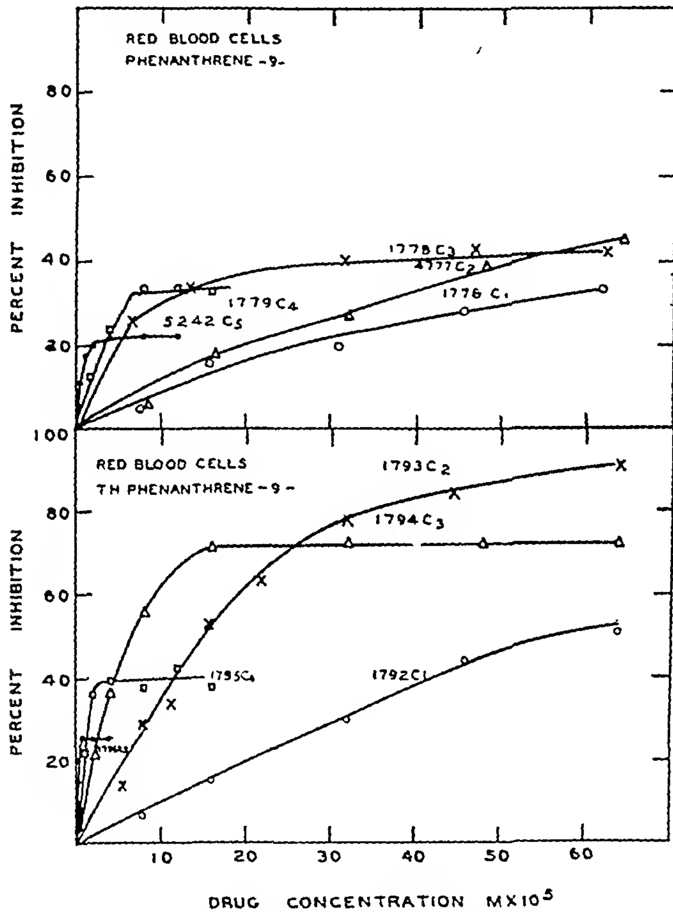

FIG. 5. THE PER CENT INHIBITION OF HUMAN RED BLOOD CELL CHOLINESTERASE BY PHENANTHRENE AMINO ALCOHOLS

The drug number (table 2) and the number of carbons of the alkyl group is given for each curve.

close relationship between some toxic symptoms of antimalarials, such as gastro-intestinal and central nervous system disturbances, and their inhibitory effect on cholinesterase.

Alles and Hawes (5) found that the cholinesterase of the red blood cells differs

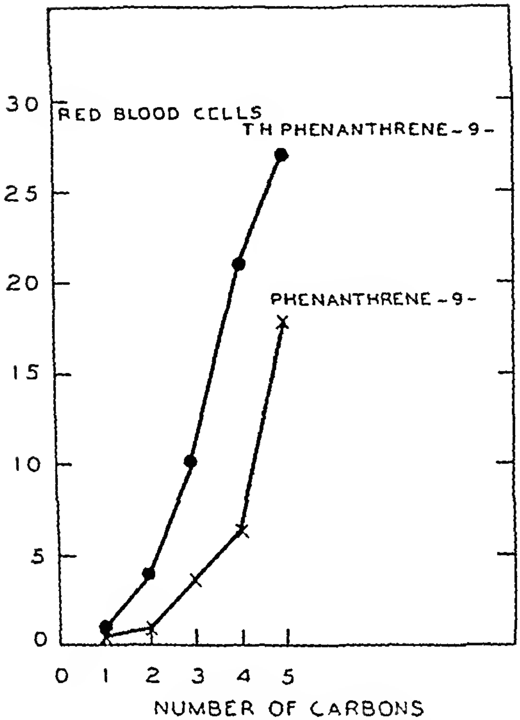

FIG. 6. THE VARIATION IN THE INHIBITION OF HUMAN RED BLOOD CELL CHOLINESTERASE BY PHENANTHRENE AMINO ALCOHOLS AS A FUNCTION OF THE NUMBER OF CARBONS OF THE ALKYL GROUP

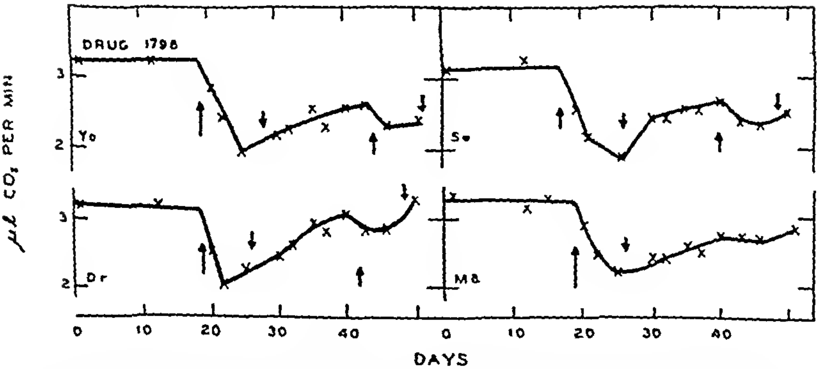

FIG. 7. THE DECREASE IN THE CHOLINESTERASE ACTIVITY OF THE WHOLE BLOOD FROM MALARIAL PATIENTS AFTER ADMINISTRATION OF DRUG #1796 (TABLE 1)
Daily doses were started at arrows pointing up and stopped at arrows pointing down.

from that in the serum in being inhibited by an excess of substrate. This has been confirmed by other workers (7, 8). Mendel, Mundell and Rudney (9) claim that the two types of enzyme can be quantitatively estimated by the use of acetyl-β-methylcholine and benzoylcholine as substrates but this has been questioned (10). The relative sensitivity of the enzyme from the two sources to inhibition by the amino alcohols (tables 1 and 2) is further proof of their dissimilarity. Some of the compounds listed in table 1, for example, #1778 and #12984, exhibit their maximum inhibitory effect on the plasma enzyme at 10^{-6} molar but require ten times this concentration to appreciably inhibit the red cell enzyme. These compounds might therefore be used as a means of determining the type of cholinesterase or of separating the two esterase activities where they exist in the same tissue. On this basis approximately ten per cent of the cholinesterase activity of human plasma is due to the type of enzyme found in red cells (table 1). Quinidine has been found to inhibit the enzymes from the two sources at concentrations comparable to compounds #1778 and #12984 and is more readily available.

SUMMARY

Phenanthrene amino alcohols were found to be very effective inhibitors of the cholinesterase of human blood plasma. The inhibition of the enzyme is related to the size of the dialkylamino group, passing through a maximum with the dipropylamino derivative in each of four homologous series. The inhibition of the plasma enzyme is decreased by hydrogenation, chlorination or hydroxylation of the phenanthrene nucleus, by replacement of the alcoholic hydroxyl group or by shifting the alkamine side chain from the 9 to the 3 or to the 2 position of the phenanthrene nucleus. Propanolamino compounds are more effective inhibitors of the plasma enzyme than their ethanolamino anologs.

Much higher concentrations of the phenanthrene amino alcohols are required to inhibit the red cell enzyme than the plasma enzyme. This offers a possible means of identifying or separating the two types of enzymic activity. Chemical changes that increase the inhibition of the plasma enzyme decrease the inhibition of the red cell enzyme.

Neither antimalarial activity in the chick nor the dose tolerated by the chick are correlated with the inhibition of cholinesterase by the phenanthrene amino alcohols.

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MYOTROPIC SPASMOLYTIC, HISTAMINOLYTIC, AND ATROPINE-LIKE ACTIONS OF SOME DERIVATIVES OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL[1])

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From the Research Laboratories, Parke, Davis and Company, Detroit, Michigan

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Recently the histamine-antagonizing and anaphylaxis-protecting actions demonstrated in these laboratories (2, 3) for dimethylaminoethyl benzhydryl ether hydrochloride (1), have shown evidence of considerable and unique clinical usefulness in various allergic states (4–10). Pursuing the possibility of improvement in potency and therapeutic range, Rieveschl (1) has continued the synthesis of related compounds.

It is the purpose of this report to give some preliminary data on certain of these compounds: new salts of the same base which appear to be histaminolytics as potent as the hydrochloride with a suggestion of lesser toxicity, and quaternary ammonium salts which show an interesting degree of atropine-like action in addition to histaminolytic potency.

Data on certain reference drugs are included for comparison, to represent a range of antispasmodic actions from predominantly "myotropic" to predominantly "neurotropic" or histaminolytic.

The abilities of the compounds to prevent fatal histamine shock in guinea pigs when injected intraperitoneally 15 minutes before the test were determined by an adaptation of the histamine-aerosol technique (for references, see Halpern (11)) used in this laboratory by Loew et al (2). The dose which protects 50 per cent of animals at an histamine exposure fatal to 95 per cent of all concurrent control animals is estimated on log. dose-probit charts (12). This differs from the previous practice of attempting quantitative estimates based on minimal statistically significant protections against death (2), wherein only partial quantitative use is made of the data and at which levels of effect statistical precision is low.

The abilities of the compounds to prevent in vitro spasms of guinea-pig ileal strips elicited with barium chloride, acetylcholine or histamine, were determined by adding varying amounts to the Magnus muscle bath one minute before addition of the spasmogenic agent and recording the resulting percentage reduction of a pre-determined standard spasm (cf. Staub (13)). A modified aerated Tyrode's solution was used. The regressions on log. dose of percentage reductions of spasm were studied by analysis of covariance (14, 15). Data from doses obviously outside the range of linear regression were excluded from the analysis. Since in the total of 944 remaining trials the mean reduction of spasm was 69.2 per cent, we have tabulated the doses forecast from the regressions for a standard 70 per cent spasm reduction in order to minimize the average error in comparison

[1] "Benadryl" is a trade name for dimethylaminoethyl benzhydryl ether hydrochloride.

from that in the serum in being inhibited by an excess of substrate. This has been confirmed by other workers (7, 8). Mendel, Mundell and Rudney (9) claim that the two types of enzyme can be quantitatively estimated by the use of acetyl-β-methylcholine and benzoylcholine as substrates but this has been questioned (10). The relative sensitivity of the enzyme from the two sources to inhibition by the amino alcohols (tables 1 and 2) is further proof of their dissimilarity. Some of the compounds listed in table 1, for example, #1778 and #12984, exhibit their maximum inhibitory effect on the plasma enzyme at 10^{-6} molar but require ten times this concentration to appreciably inhibit the red cell enzyme. These compounds might therefore be used as a means of determining the type of cholinesterase or of separating the two esterase activities where they exist in the same tissue. On this basis approximately ten per cent of the cholinesterase activity of human plasma is due to the type of enzyme found in red cells (table 1). Quinidine has been found to inhibit the enzymes from the two sources at concentrations comparable to compounds #1778 and #12984 and is more readily available.

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[1] "Benadryl" is a trade name for dimethylaminoethyl benzhydryl ether hydrochloride.

of equivalent doses. It must be emphasized that in view of the use of a standard one-minute exposure of the intestinal strip to the compounds, these data represent a combination of speed of penetration to site of action with intrinsic intensity of prophylactic action against spasm, which seems a fair measure of potency.

Examination of the scattergrams of the 48 sets of data (16 compounds, 3 spasm types) revealed no evidence of non-linearity; indeed, cases of least scatter showed sharply a straight-line relation. The pooled correlation coefficients were 0.768 with 270 degrees of freedom for acetylcholine, 0.598 with 239 d.f. for histamine, and 0.329 with 339 d.f. for $BaCl_2$, all very highly significant. Differences among these coefficients reflect our confirmation of the experience of others with the relatively erratic nature of data with $BaCl_2$ as compared with histamine and acetylcholine, particularly the latter. In terms of this uncontrolled variability in the data, the log. dose-effect slopes for the 16 compounds against $BaCl_2$ did not differ significantly ($F = 1.50$, with 15 and 339 degrees of freedom), so that the average regression (26.13 added per cent prevention of spasm per unit log. dose) was used as the best estimate for forecasting individual "70% effective log. doses" (log. ED_{70}'s)against $BaCl_2$. In the case of the histamine data, slopes differed with significance (5% level) when all 16 drugs were considered; but those for the eleven drugs with considerable histaminolytic specificity (1571 F and all benzhydryl ethers) were statistically homogeneous ($F = 1.21$ with 10 and 94 d.f.), so that their average regression (50.71 per cent per unit log. dose) was used in forecasting respective log. ED_{70}'s. In the case of acetylcholine, slopes of the 16 drugs differed more significantly (better than 1% level), but those of the 10 ethers and Pavatrine constituted a homogeneous group ($F = 1.11$ with 10 and 215 d.f.), whose average regression (41.52 per cent per unit log.) was used in respective log. ED_{70} estimates. In all other cases individually experienced regressions were honored. "Approximate" standard errors (16) of the log. ED_{70} forecasts were computed for estimating "68% fiducial limits".

Acute lethal toxicities were determined in white rats by the intraperitoneal route. Median lethal doses and their approximate standard errors were estimated graphically (12). Data on groups of the compounds were collected at different times from animals of different lots but of comparable size (100–150 grams).

All doses are expressed as μgm. or mgm. of "Benadryl" to facilitate molecule-for-molecule comparisons while retaining the advantages of per-weight potency expressions for "Benadryl". Pharmacologically active equivalents of aminophyllin and atropine sulfate were regarded as 0.5 M, even though the ethylene diamine of the former may possibly make some contribution to action.

Results and Discussion. Results are summarized in table 1.

The in vitro potencies of aminophyllin are all so low as to make such data useful controls over the possibility of non-specific explanations of the more striking results. Its effectiveness in histamine shock in vivo, even though of relatively low order, seems out of line with its direct spasmolytic potencies if information may be tentatively transposed from the gut to bronchial musculature. One is inclined to attribute its in vivo effectiveness to some action not present with relative significance among the other compounds listed (cf. Lehmann and Young (17)).

In table 2 the various drugs have been arranged in average logarithmic orders

of significantly increasing in vitro molar potencies against the three spasmogenic agents.

In prevention of $BaCl_2$ spasm, commonly believed due to action close to the fundamental "contractile mechanism", the two synthetic esters Pavatrine and Trasentin are superior. The three salts of the tertiary Benadryl base (compounds 1–3) as a class are significantly less potent, while the various salts of the Benadryl quaternary bases (compounds 4–10) drop to the general class of papaverine.

In preventing the acetylcholine spasm, atropine retains its classical superiority. Pavatrine is inferior to atropine with statistical reliability especially when its less steep log. dose-effect slope is considered. Interestingly, the two dimethyl ethyl benzhydryloxethyl ammonium salts (compounds 9 and 10) were not significantly less potent than Pavatrine, whereas the trimethyl ammonium compounds (compounds 4 to 8) as a class were somewhat less potent. The various salts of the Benadryl tertiary base (compounds 1 to 3) were significantly less potent than either Trasentin or Pavatrine when pitted against acetylcholine.

In ability to prevent histamine spasm, the three salts of the tertiary Benadryl base did not differ significantly among themselves. The quaternary ammonium salts did not form a homogeneous potency group, but experienced differences are at present difficult to interpret. Some were as potent, some apparently less potent that the tertiary type. Non-specific anionic influences may be present. In general, both the tertiary and quaternary bases were far more potent than the diamine, 1571F. From the latter, "specificity" was progressively lost in passing through Pavatrine, Trasentin, atropine, papaverine and aminophyllin.

The lack of correlation among anti-barium, anti-acetylcholine, and antihistamine actions of these drugs is apparent. Not only atropine but also histamine may act more peripheral to the fundamental contractile mechanism than does the barium ion. First apparent in European studies of the Fourneau compounds e.g., 1571), it now seems emphatically fallacious to consider histamine stimulation as "myotropic" in the sense that barium stimulation is It seems, furthermore, imperative to consider different remote points of attack ("receptor substances") for acetylcholine and histamine. Individualities of log. dose-effect regression coefficients of the drugs, as pitted against the three respective spasmogenic agents, bear interestingly on these points (cf. above).

There is reasonably good correlation between in vitro and in vivo "anti-histamine" potencies of the drugs. However, some salts of the Benadryl quaternary bases appeared more potent in preventing systemic histamine shock than did those of the tertiary type, without correspondingly superior in vitro potencies. This is tentatively attributed to the greater atropine-like action of the quaternary compounds, effective against a cholinergic factor in histamine shock. Existence of such a factor, while controversial, is not without evidence (e.g., Frommel, Aron, Herschberg, Piquet and Goldféder (18)). It is recognized, however, that differences in affinities among tissues, or fundamental actions of the quaternary bases not yet known may contribute to their superior effectiveness against systemic histamine shock in the guinea pig. Actions at many other sites and in other species will need study.

TABLE 1

*Effective doses of reference drugs and certain benzhydryl alkamine ethers, expressed as μgm. or mgm. of "Benadryl"**

COMPOUND	DOSE PER 100 CC MUSCLE BATH, FORECAST FROM REGRESSION, FOR 70% REDUCTION OF ILEAL SPASM DUE TO: 10 mgm $BaCl_2$	2 μgm acetylcholine bromide	8 μgm histamine diphosphate	PROPHYLACTIC LD_{50} AGAINST HISTAMINE-AEROSOL DEATHS I P GUINEA PIGS (AS MGM OF BENADRYL PER KGM)†	7-DAY LD_{50} I P WHITE RATS (AS MGM OF BENADRYL PER KGM)†
	(as μgm of Benadryl, with 68% fiducial limits† in parentheses)				
Reference compounds					
I. Theophylline ethylenediamine dihydrate (Aminophyllin)	3,700,000§ (110,000–120,000,000)	64,000 (55,000–74,000)	82,000 (68,000–100,000)	>64	313 ± 13
II. Papaverine hydrochloride	3100 (1900–5200)	650 (580–730)	660 (550–790)	>19 (?)	
III. Atropine sulfate monohydrate	3500¶ (1300–9600)	0.3 (0.2–0.4)	65 (52–82)	>13 (?)	244 ± 12
IV. β-Diethylaminoethyl fluorene-9-carboxylate hydrochloride (Pavatrine)	‡ 98 (73–130)	0.7 (0.6–0.8)	‡ 37 (29–46)	>42	211 ± 10
V. β-Diethylaminoethyl diphenylacetate hydrochloride (Trasentin)	220 (150–320)	7.6 (7.0–8.3)	43 (37–50)	>47	176 ± 16
VI. N-Diethylaminoethyl ethyl aniline hydrochloride (1571 F)	120,000 (13,000–1,100,000)	1000 (770–1300)	16 (11–22)	6.4 ± 1.1	161
Benzhydryl ethers $(C_6H_5)_2CHO\cdot CH_2\cdot CH_2\cdot R$					
1.—$N(CH_3)_2\cdot HCl$ (Benadryl)	340 (220–520)	15 (14–16)	1.1 (1.0–1.2)	1.8 ± 0.3	61 ± 9
2.—$N(CH_3)_2\cdot HOOC\cdot CH_2\cdot CH_2\cdot COOH$	‡ 470 (180–1200)	‡ 14 (12–15)	‡ 0.5 (0.1–3.6)	‡ 2.0 ± 0.3	123 ± 9
3.—$N(CH_3)_2\cdot HOOC\cdot COOH$	1300 (530–4000)	12 (8–17)	1.1 (0.9–1.3)	2.7 ± 0.6	109 ± 9

1.—$N(CH_3)_3\ Cl$	1800 (800–1200)	2.1 (1.6–2.7)	2.2 (2.0–2.4)	0.9 ± 0.3	42 ± 5
5.—$N(CH_3)_3 \cdot Br$	4800 (1500–16,000)	1.5 (1.3–1 8)	1.1 (0.5–2.3)		83 ± 15
6.—$N(CH_3)_3 \cdot I$	2300 (?)	2.3 (1.7–3.1)	3.2 (3 2–3.4)	0.5 ± 0.3	43 ± 5
7.—$N(CH_3)_3 \cdot O \cdot SO_2 \cdot OCH_3$	‡ 980 (380–2500)	‡ 2.1 (1 5–2 9)	1.2 (0 9–1 8)	1.3 ± 0.7	34 ± 8
8.—$N(CH_3)_3 \cdot O \cdot SO_2 \cdot C_6H_4 \cdot pCH_3$	3300 (1500–7000)	3.3 (3.1–3.6)	3.6 (2.8–4.7)	2.8 ± 0.4	47 ± 5
9.—$N(CH_3)_2(C_2H_5) \cdot Br$	6200 (3200–12,000)	1.1 (1.0–1.2)	3.1 (2.8–3.4)	1.8 ± 0.4	38 ± 10
10. —$N(CH_3)_2(C_2H_5) \cdot O \cdot SO_2 \cdot C_6H_5$ (CH_3)	1600 (930–2000)	0.8 (0.6–1.0)	2.6 (2.3–2.9)	1.7 ± 0.3	34 ± 4

* Derived from actual effective weights by the factor

$$\frac{\text{equivalent weight of "Benadryl"}}{\text{equivalent weight of respective compound}}$$

Equivalents were taken as molecular weights except in the cases of the (diacidic) theophylline ethylenediamine and the (dibasic) atropine sulfate, where 0.5 M was taken as an equivalent.

† ± approximate standard error.

‡ Differences not statistically significant (5% level) within any one bracket, by analysis of covariance.

§ Hypothetical forecast; experimental range did not actually extend to 70% prevention of spasm.

¶ Hypothetical; spasmolytic regression appeared to fail, short of 70% prevention of spasm, due to emergence of an opposing action at about 1600 µgm./100 cc.

Certain relationships between chemical structure and action are illustrated. It is becoming increasingly apparent that two-carbon alkylamines (first classical for adrenergic activators (19)) are of primary importance in physiology and pharmacology of the autonomic system and many other biological mechanisms. Among such compounds are included activators, blockers, and others which interfere with enzymatic attack on activators, in the adrenergic, cholinergic and histaminic biological systems. From this point of view it is not too surprising to find classical borderlines of selectivity of action among the three systems breaking down in certain series of substituted ethylamines such as the present ones[2,3].

TABLE 2

Drugs arranged in (average logarithmic) orders of significantly increasing molar potencies
(At 70% spasm reduction level)

IN VITRO AGAINST:		
$BaCl_2$ (10 mgm.)	Acetyl Choline·Br (2 μgm.)	Histamine diphosphate (8 μgm.)
0.0 Aminophyllin	0.0 Aminophyllin	0.0 Aminophyllin
1.5 1571 F	1.9 {1571F, Papaverine}	2.1 Papaverine
3.1 {Papaverine, Atropine*, "Quaternary" ethers}	3.7 "Tertiary" ethers	3.2 {Atropine, Trasentin, Pavatrine}
3.8 "Tertiary" ethers	3.9 Trasentin	3.7 1571 F
4.4 {Trasentin, Pavatrine}	4.5 Trimethyl "quaternaries"	4.6 "Quaternaries"
	5.0 {Dimethyl ethyl "quaternaries", Pavatrine}	5.1 "Tertiaries"
	5.3 Atropine	

* Hypothetical; actually not attained due to opposing action at higher doses.

The resultant of influences of molecular structure at both ends of the 2-carbon

[2] The 2-carbon bridge can be traced in such heterocyclic structures as arecoline with muscarinic action, naphthyl-(1')-methylimidazoline or Privine with "adrenergic" and "cholinergic" actions (20), and certain Fourneau aryloxethylamines and benzodioxanemethyl amines (nitrogen to oxygen bridge) with actions in the histaminic and adrenergic systems. Certain well-known exceptions to the ethylene bridge occur where a cyclic structure is involved, as in atropine, pilocarpine, eserine and neostigmine. In such cases cyclization may provide the equivalent of an ethylene bridge.

[3] Since preparation of this paper a relevant discussion by Dawes (Brit. Med. J., 1946, 43) has come to our attention. The author points out a tendency for parallelism among a) lengthening of auricular refractory period (quinidine-like action), b) local anesthetic action, and c) spasmolytic action among substituted ethylamines. The simultaneous possession of the latter two actions by many drugs has been known. An aromatic nucleus in opposition to the basic part of the molecule seems important. Whether the linkage be ester, ether or carbinol "does not seem to be of the first importance".

bridge appears to aid in determining the biological system affected and the quality of effect. So far as present information goes, in the adrenergic system an optimal circumstance of the aminoethyl chain appears to be extension or attachment to the aryl structure; in the histaminic system, attachment to a heterocyclic structure for activation or through an ether or N linkage to an aryl (Fourneau's two original types) or aralkyl group for interference; in the cholinergic system, either ester or ether linkage, with quality and quantity of action influenced by the state of both molecular extremities. For example, loss of the cyclic tropine structure in the basic moiety of atropine usually results in compounds with markedly reduced interfering action in the cholinergic system (but, according to the acidic moiety, commonly increased "myotropic" spasmolytic action as in the case of Trasentin or Pavatrine above and/or histaminolytic action as in certain cases studied by Lehmann and Knoefel (21)). When the acid part is changed slightly, as from the diphenylacetyl of Trasentin to the fluorene-9-carboxylate of Pavatrine (see above), remarkable strides can be made back toward atropine's higher cholinergic-system potency (with maintenance or still further improvement of the presumably independent "myotropic" action). Starting again with Trasentin, if the carboxyl group with its ester linkage is replaced by an ether function and the N-ethyl groups replaced by N-methyl, one has the "Benadryl" type with still further reduction in cholinergic-system (and some relinquishing of "myotropic") actions, but with the remarkable appearance of high interfering potency in the histaminic system. Substitution of the N-ethyl groups of Trasentin by N-methyl accomplishes no such change in properties, but this substitution *along with* change in type of linkage is essential for "Benadryl's" high potency (2) and N-methyl substitution has also improved the Fourneau diamines as anti-histamine agents (Halpern (11)). Finally, conversion of "Benadryl", a tertiary amine, to trimethyl quaternary salts (4 to 8 of the table) does not alter histaminic-system action to a remarkable degree but does restore anticholinergic action of these *ethers* almost to the degree that changes in the acidic moiety did in the *ester* series (cf. Pavatrine *vs.* Trasentin) and at the same time still further reduces "myotropic" action. Substitution of one N-methyl group with ethyl (compounds 9 and 10) was seen to provide additional atropine-like power.

Within the general thesis of interactions of groups on an ethylamine in determining selectivity and quality of action among the adrenergic, cholinergic and histaminic systems, the present data also yield points of more specific theoretical interest.

The mode of action of "Benadryl" appears to be a competitive blocking of histamine at the effector cell (22). Comparing the structure of "Benadryl" with that of histamine, the 2-imidazole structure of the latter is replaced by a benzhydryl ether group, and as the function of the nitrogen is changed from primary to secondary and to tertiary (Benadryl), the histamine interference increases (2). Thus, in the presence of the benzhydryl ether group, as histamine-interference increases the function of the nitrogen progressively *deviates* from that in histamine. From this view it is not so surprising to find essential maintenance of interfering potency in passing on to the present quaternary amines. But now one crosses

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into the territory of choline derivatives and there has been a rebirth of action in the cholinergic system, of interfering rather than activating type. It would appear that the benzhydryl (aralkyl) ether nucleus assures non-activation in both the histaminic and (muscarinic) cholinergic systems. It requires, through its contributions to the molecule as a whole, di- or tri-N-alkylation (*deviating* from histamine) for affinity in the histaminic system, but a quaternary ammonium structure (*conforming* to choline) for better affinity in the cholinergic system.

For comparison of the atropine-like action of the present ethers with that of analogous known esters, certain properties of the latter must be pointed out. Although the quaternary ammonium structure is necessary for noteworthy cholinergic activation, atropine, the classical interfering agent in the "muscarinic" portion of this system, is a tertiary base. As judged most commonly by mydriasis, it has become increasingly apparent since the work of Jowett, Pyman and Dale (see Ing, Dawes and Wajda (23)) that in such esters of tertiary basic alkanols an aryl structure in the acid moiety meets a requirement for reasonably good atropine-like action, and di-substitution preferably to mono (24, 23). It now appears that the tertiary bases patterned after atropine have been a step behind in optimal intrinsic potency; a variety of aromatic substituted acid esters of tertiary basic alkanols, including atropine, are now being found to have greater intrinsic interfering potency in cholinergic systems when converted to quaternary ammonium salts; i.e., to a closer formal relationship with the choline *activators* of the system (see Ing *et al.* (23) and Blicke (24) for data and references). "Myotropic" potency may concurrently decrease and toxicity increases. It is argued that atropine-like as well as curariform interference with the acetylcholine onium cation are importantly dependent upon the onium ion of the interfering agent, being optimal when that ion is stabilized in quaternary ammonium salts (23).

With activators and interferers in the cholinergic system thus tentatively brought to common grounds at the basic moiety of the molecule, interest centers in the other part. Jowett and Pyman's aryl acid requirement for the tertiary amines as concerns atropine-like action, is fulfilled in the newer quaternary type (23). The requirement may be optimal in the latter. This thought is pointed up by the complete swing from specific activator to presumably specific interferer in going from simple alkyl acid esters of choline to aryl substituted acid esters. However, while aryl groups meet a requirement in this manner, they are apparently not exclusive in their ability to meet it. Conversion of carbamylcholine, a potent cholinergic activator, to an atropine-like drug has recently been accomplished by di-butyl substitution on the carbamyl N (25, 26, 27). Di-*n*-butylcarbamyl- and arylacylcholines thus constitute the two known types of choline esters which interfere with the simpler analogous alkylacylcholine activators.

The case of the present ethers is, so far as can be seen now, completely analogous to the case of atropine-like action of alkamine esters just outlined. The salts of the tertiary ethylamine benzhydryl ether exhibit appreciable atropine-like action (see tables), but are inferior to the nearly analogous ester, Trasentin,

presumably in part because of departure of the ether linkage from the ester form of the activator. Jowett and Pyman's aryl requirement is met, though in an ether instead of an ester. Analogously with the esters, conversion to quaternary ammonium salts, still meeting the aryl requirement, increases the atropine-like potency several fold while decreasing the "myotropic" action and increasing toxicity. One would predict, however, that when compared quantitatively with the corresponding ester they will be found of lesser potency. Analogously to swinging from activators to interferers in the esters, one contrasts the known cholinergic (muscarinic) activating action of simple akyl ethers of choline (methyl and ethyl) with the reasonably potent interfering action of the present aralkyl (benzhydryl) ethers. The analogue in the ether series of di-*n*-butylcarbamylcholine of the ester series is at present lacking.

There is one further parallel between the esters and ethers. Ing *et al.* found that in their benzilic acid esters of quaternary ethanolamines, atropine-like action was sharply increased by substituting one of the three N-methyl groups with an ethyl. This is analogous to the present finding that the two dimethyl ethyl benzhydryloxethyl ammonium salts (9 and 10 of table) are significantly more potent against acetylcholine than the trimethyl compounds (4 to 8). There is a strong suggestion (compound 9 *vs.* 5) that the reverse is true in preventing histamine spasm.

Failure of the present experiments to show any remarkable difference in histamine-interfering potency between the tertiary and quaternary compounds suggests that the ionic state is not of the order of importance that it is in the cholinergic system.

As an anticipated parallel with the aralkyl acid esters of quaternary ethanolamines a study of possible nicotinic and curariform actions of the present analogous ethers will be in order. At present there are only suggestions implied by the observation that while the tertiary amines were severe clonic convulsants at toxic doses in the rats, the quarternary types were ataxant-depressant (curariform?) before becoming terminally convulsant, and in general more lethal. The suggestion of increased lethal toxicity of compounds 9 and 10 with one N-methyl group substituted by ethyl may be related to the known curariform action of tri-ethylammonium ethers and the interesting synaptic-blocking action of the tetra-ethyl ammonium ion currently being studied by Moe (28).

The data suggest a possible improvement in therapeutic range of "Benadryl" in the acid succinate, but a variety of tolerance studies will be necessary to prove this possible anionic influence. One will expect the therapeutic addition of an atropine-like action over and above "Benadryl's" in the quaternary compounds. This may be an asset in therapy of certain allergic states, and a predominantly troublesome side-effect in others. The quaternary compounds do not appear promising as *general* visceral spasmolytics because of a lack of good myotropic action to accompany the atropine-like action. Even the latter action is lower by the present test than atropine's and is accompanied by far greater lethal toxicity in species used so far.

In line with Lehmann's (29) suggestion on the possible usefulness of a combina-

tion of anti-histamine and atropine-like actions at the uterus, studies of the Benadryl quaternary bases in this field may be of eventual interest.

SUMMARY

On the isolated quinea pig ileum and in the intact animal the acid succinate and acid oxalate of β-dimethylaminoethyl benzhydryl ether have an order of histaminolytic action similar to the hydrochloride's, but the acid succinate may be less toxic (molecular comparisons). This suggestion of an anionic influence should be studied further.

Quaternary ammonium derivatives of the base, while entering the class of choline derivatives, retain potent histaminolytic properties and at the same time exhibit considerably more potent atropine-like action than salts of the tertiary type (in contrast with the known *activating* action of lower alkyl choline ethers). The added atropine-like action appears to increase their efficacy in protecting intact guinea pigs against death from inspired histamine mist It is accompanied by diminished "myotropic" action and increased toxicity.

The importance of 2-carbon alkylamines in the histaminic and cholinergic as well as adrenergic systems, and the breaking down of classical boundaries of specificity in these systems concurrently with interactions between changing molecular groups are illustrated. Parallels in the relation of structure to action are drawn between the present alkamine ethers and known analogous esters.

Acknowledgment The present tabulation of data represents analysis of an accumulation to date in this laboratory. They therefore include some collected by two of us (2, 30, 31) in collaboration with Dr. E. R. Loew, now located at the University of Illinois Medical School We are grateful to Dr. F. F. Yonkman, of Ciba Pharmaceutical Products, Inc , for supplies of crystalline Trasentin; to Dr. R. R Burtner of G. D Searle and Co. for crystalline Pavatrine; and to Mr. M L. Black and Dr. G. Rieveschl, Jr , of these laboratories, for 1571F and the benzhydryl ethers, respectively

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ANESTHESIA

XXII. The Anesthetic Action of Isopropyl Methyl Ether[1]

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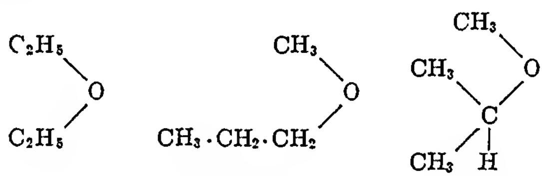
In a former communication (1) to this journal the authors demonstratrated the availability of n-propyl methyl ether as an anesthetic. Since that time more than one hundred successful clinical anesthesias have been conducted with n-propyl methyl ether. A clinical study of anesthesia under n-propyl methyl ether has been recorded elsewhere (2). Accordingly it occurred to us to study the anesthetic properties of another isomer of ethyl ether, namely, isopropyl methyl ether. The spacial relationship of the three isomers is apparent from the following formulas:



Ethyl ether | n-Propyl methyl ether | Isopropyl methyl ether

Our survey of the literature revealed no comprehensive studies with isopropyl methyl ether as an anesthetic agent. Marsh, working in Leake's laboratory, studied the compound among other substances in correlating density and anesthetic activity (3).

Isopropyl methyl ether is a volatile, colorless liquid with a characteristic mild camphoraceous odor; the boiling point is 31.2°C. and the specific gravity 0.720 at 15.6°C.

Anesthesia in the monkey. Three large *Macacus rhesus* monkeys were anesthetized with isopropyl methyl ether. The technic is described in detail in our former studies (4). The induction period was comparable to that with ethyl ether. Surgical anesthesia was uneventful; breathing was stertorous, deep and regular. Recovery from anesthesia was prompt; more rapid than with ethyl ether. There was little excitation during the recovery period. The quantities employed were about equal to the amounts of ethyl ether used to produce similar anesthetic syndromes.

Anesthetic index (dog). The dogs employed were, as far as possible, of uniform

[1] The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Company of Cleveland, Ohio.

weight. They were fed a diet of "Purina Chow" for one week prior to the experiment and fasted 12 hours immediately before anesthetizing. At least 2 day intervals elapsed between anesthesias with the same animal. The procedure was identical with that employed in our cyprome ether studies (4). The number of cubic centimeters of the agent per kilogram required to produce surgical anesthesia was divided into the volume required to produce respiratory arrest. The quotient was designated as the anesthetic index. The results are summarized in table 1.

These results indicate that isopropyl methyl ether and its isomers ethyl ether and n-propyl methyl ether exhibit anesthetic indices of the same order of magnitude. From the volumes required for induction, isopropyl methyl ether appears to be slightly less potent than ethyl ether and approximately 75 per cent as potent as its isomer n-propyl methyl ether.

TABLE 1

DOG NO.	SEX	WEIGHT	INDUCTION	RESPIRATORY FAILURE	ANESTHETIC INDEX
		kgm.	*cc /kgm*	*cc /kgm*	
1	F	5.0	1.10	2.90	2.63
2	M	5.5	1.00	2.82	2.82
3	M	7.1	1.13	2.95	2.62
4	F	5.4	1.10	2.59	2.36
5	F	7.3	1.23	2.19	1.78
6	M	6.5	1.24	2.71	2.19
7	F	5.2	1.26	2.72	2.16
8	M	6.6	0.98	2.58	2.63
9	F	5.1	1.08	2.25	2.08
10	F	6 8	1.03	2.50	2.42
Mean			1.12	2.62	2.37

Blood pressure studies (dog). The effect of isopropyl methyl ether on the blood pressure was determined by anesthetizing the animal with ethyl ether. The blood pressure was determined in the usual manner by cannulating the carotid artery. The anesthetic reservoir containing ethyl ether was removed to allow the animal to exhale much of the ether, then isopropyl methyl ether was substituted for the ethyl ether. The respiratory tracings were made by means of a tracheal cannula and rubber tambour. The anesthesia was deepened to the point of repiratory collapse. The animal was then allowed to recover. The experiment was carried out on four animals and a typical tracing is shown in chart 1.

Electrocardiographic studies (dog and monkey). Three monkeys and two dogs were cardioscoped under isopropyl methyl ether at various planes of surgical anesthesia. After surgical anesthesia of 20 minutes duration permanent tracings of the E.C.G. were made of three monkeys. A typical tracing Lead II is shown in chart 2 of the monkey before and under surgical anesthesia. Changes in rate

of heart beat were insignificant, the R-spike was slightly depressed in amplitude; the T-wave was neither inverted nor flattened.

Effect on the perfused heart (frog). Isopropyl methyl ether was dissolved in Howell-Ringer's solution and perfused through the frog's heart *in situ.* Ten experiments with different concentrations of the ether were carried out. Concentrations of 0.014 molar (100 mgm. per cent) were devoid of effect. Solutions containing 1000 mgm. per cent promptly depressed the rate and amplitude of the heart beat. A typical tracing is shown in chart 3.

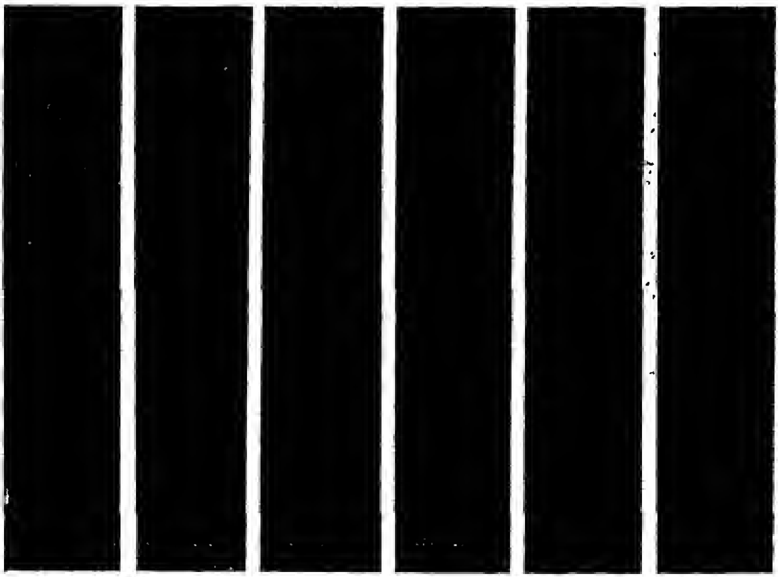


CHART 1. BLOOD PRESSURE OF DOG UNDER ISOPROPYL METHYL ETHER ANESTHESIA

The upper tracing is respiration: (1) ethyl ether anesthesia with tracheal cannula, (2) removal of ethyl ether for 2 minutes, (3) 5 minutes anesthesia with isopropyl methyl ether with tracheal cannula, (4) 22 minutes anesthesia with isopropyl methyl ether (deep surgical anesthesia), (5) 35 minutes anesthesia with isopropyl methyl ether (threatened respiratory collapse), (6) 40 minutes after beginning anesthesia (recovery).

Liver function tests (dogs). Four dogs were subjected to the bromsulfalein liver function test as set forth in our studies with cyprethylene ether (5). The dye excretion period was 30 minutes. Twenty-four hours after 60 minute anesthesias with isopropyl methyl ether, the rate of dye excretion was not significantly different from the preanesthetic values.

Blood urea studies (dog). Three dogs were anesthetized to the surgical plane and then given more of the anesthetic until respiratory arrest occurred according to the anesthetic index technic. Prior to anesthesia and 24 hours later blood samples were drawn for analysis. No significant changes in blood-urea nitrogen were observed.

Fate in the body. The U.S.P. test for methanol in ethanol was adapted to the Folin-Wu blood filtrate. The anesthetic in blood gave a negative test. One-tenth mgm. methanol in 1 cc. of blood gave a definite positive test. With the bloods of 2 dogs and 2 monkeys, after one hour anesthesia with isopropyl methyl ether, the test for methanol was negative.

Delayed anesthetic deaths. Ten adult rats were anesthetized with isopropyl methyl ether to the surgical plane and maintained in this state for 30 minutes.

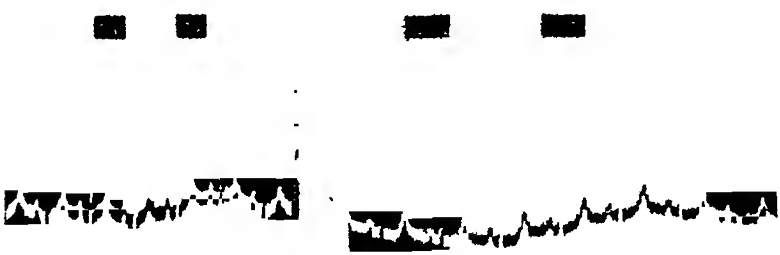


CHART. 2. ELECTROCARDIOGRAMS, NORMAL AND UNDER ISOPROPYL METHYL ETHER ANESTHESIA FOR 30 MINUTES (MONKEY LEAD II)
Normal Anesthesia

CHART 3 EFFECT OF ISOPROPYL METHYL ETHER ON THE FROG'S HEART

1) Howell-Ringer's solution, fast drum; 2) same, slow drum, 3) 0.08 molar isopropyl methyl ether; 4) same after 7 minutes; 5) same with fast drum.

Five animals were sacrificed at the end of 2 weeks and no significant findings were observed in the liver or kidneys. At the end of 3 weeks none of the remaining animals had died or appeared to be in an unhealthy condition.

Clotting time and hemolysis. The clotting time of the blood was determined in 3 normal dogs by the capillary tube method. The clotting time was between 55 and 70 seconds. Within the error of the experiment this period was neither diminished nor increased under surgical anesthesia with isopropyl methyl ether.

Volumes of 10 cc. of isopropyl methyl ether in varying concentrations in normal

salt solution were maintained at 28° C. The red cells were supplied by adding 0.1 cc. defibrinated dog's blood. No hemolysis occurred over a 24 hour observation period in concentrations of 50, 100 and 150 mgm. per cent. A saturated solution produced hemolysis within 2 hours.

Histological studies of viscera (rat, dog and monkey). Five of the rats used in the delayed anesthetic death experiments were sacrificed and their livers and kidneys were found to be free from significant changes. Six dogs were anesthetized for 60 minutes each on 3 alternate days. On the sixth day after the first anesthesia liver biopsies were performed. There were no significant findings.

Two *Macacus rhesus* monkeys were subjected to the foregoing procedures. No significant findings were observed.

Preanesthetic medication. In dogs, inducing isopropyl methyl ether anesthesia with nitrous oxide or cyclopropane-oxygen mixtures was uneventful. Preanesthetic medication with pentobarbital sodium or morphine-atropine was found to be compatible with isopropyl methyl ether anesthesia. Eight experiments were conducted on 5 animals.

Physical properties. Solubility in water: An 11 cc. volume of isopropyl methyl ether was agitated with 100 cc. of water for 2 hours at 28°C. in a "Cassia Flask". The liquids were allowed to separate for 12 hours and the volume of supernatant ether measured. The solubility was found to be 9.9 cc. per 100 cc. water. Our value for anesthetic ethyl ether is (4) 8.6 cc. and for n-propyl methyl ether is 5.0 cc. per 100 cc. (1).

Oil water coefficient: The oil/water coefficient was calculated from the data set forth by Carr et al. (6) on the relationship between water insolubility and oil/water coefficient. The value for isopropyl methyl ether is 2 ± 0.2, approximately half the value assigned to ethyl ether.

Inflammability range: Isopropyl methyl ether is isomeric with ethyl ether and will therefore have approximately the same inflammability range. This is about 2 per cent (lower limit) in air or oxygen (7).

Vapor pressure: The vapor pressure of isopropyl methyl ether determined at 26°C. in a nitrometer is 635 mm., that of ethyl ether at the same temperature is 555 mm. (8).

SUMMARY AND CONCLUSIONS

1. Isopropyl methyl ether, an isomer of ethyl ether, is a volatile liquid exhibiting anesthetic properties when administered by inhalation to various species of animals.

2. The potency of isopropyl methyl ether is approximately 25 per cent less than that of ethyl ether.

3. In the dog, isopropyl methyl ether anesthesia produces no functional liver damage as shown by the bromsulfalein test. In these experiments in the rat, dog and monkey, anesthesias with isopropyl methyl ether produced no histopathological changes in the liver and kidneys.

4. Neither the monkey's nor the dog's heart showed any significant electrocardiographic changes under anesthesia with isopropyl methyl ether.

5. The blood pressure of the dog remains essentially unaltered under anesthesia with isopropyl methyl ether.

6. This isomer of ethyl ether compares favorably with ether as an inhalation anesthetic in several species of animals. Its increased volatility appears to compensate for its diminished potency. This first approximation of the anesthetic properties of isopropyl methyl ether, in our opinion, warrants its careful and judicious trial in man by skilled anesthetists. Extensive and intensive study alone in human anesthesia will reveal whether or not this mixed ether will warrant a place in the armamentarium of the anesthetist.

Addendum. These experiments having been completed, we deemed that the properties of isopropyl methyl ether warranted its trial as an anesthetic in man. On March 22, at 4:00 P.M., one of us (J. C. K., Jr.) administered isopropyl methyl ether to an anesthetist, Constance Black, by the open drop method. The induction period was about 5 minutes. Light anesthesia was continued for 3 minutes. The recovery was rapid and uneventful. The induction period was not marked by any excitment.

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BLOCKADE OF THE NICOTINE ACTION ON THE BLOOD PRESSURE BY THIAZOLE-COMPOUNDS (SULFATHIAZOLE AND THIAMINE)

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In previous experiments on the isolated gut of the guinea pig and the rabbit, it was demonstrated that certain sulfur containing compounds such as sodium sulfathiazole, sodium sulfamerazine and sulfanilamide (1) as well as thiamine hydrochloride and thiazole (2) inhibit the contraction of smooth muscles induced by stimulation of the sympathetic synapses with nicotine. The action is reversible and is not caused by a preceding paralysis of the ganglia. In consideration of the manifold influences of nicotine on the entire vegetative nervous apparatus, it appeared important to investigate whether such inhibitory actions by the above named sulfur containing compounds could also be produced *in vivo* at the vasomotor synapses of the thoracic and abdominal sympathetic nervous system. Stimulation of these ganglia causes, as is known, a generalized arterial and arteriolar constriction with elevation of the blood pressure; the action of nicotine on these ganglia and also directly on the cells of the adrenal medulla likewise results in an increased output of epinephrine. Such an inhibition of the blood pressure raising effect of nicotine would be remarkable both from a theoretical and practical standpoint. It would open new ways for the more intimate pharmacological analysis of this important nicotine action and also offer a new method for the prevention of vasopressor effects as a consequence of ganglionic stimulation.

METHODS. These investigations were carried out in about 40 cats of the average weight of 2–3 kgm. The animals were anesthetized by intraperitoneal injection of 30–35 mgm. nembutal per kgm. body weight. In order to eliminate vagal stimulation of the heart by nicotine the cats received at the start of the experiment, and several times during its progress, 2 mgm. atropine sulfate intravenously. Because of the deep nembutal anesthesia and the atropinization, disturbing reflexes were for the most part eliminated, so that the usually employed section of the spinal cord could be dispensed with. Respiration was maintained with the help of a Starling pump. The blood pressure was recorded from the right carotid artery using a mercury manometer. The salts of the organic sulfur compounds in neutral aqueous solution were infused into the femoral vein with the help of a burette, in such a way that 1 cc. was injected per minute. Thiamine hydrochloride (Merck) was used in 0.1% solution; sodium sulfathiazole, sodium sulfadiazine, sodium sulfapyrazine and sulfanilamide were employed in 1–2% solution and para-aminobenzoic acid in a 3% solution. After infusion of various dosages of these drugs, the blood pressure raising effect of nicotine was tested by the injection of a 1:1000 aqueous solution of nicotine base into the jugular vein; for that purpose doses of 0.1, 0.2, 0.4, 0.5 and 1.0 mgm. nicotine base were employed. For the additional testing of the reaction of the nervous apparatus of circulation we employed adrenaline, acetylcholine (for its muscarine- as well as for its

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nicotine-like effect), carbaminoyl choline and acetyl-β-methylcholine in various intravenous dosages before and after the infusion of the organic sulfur compounds; likewise the response of the heart vagus to electrical stimulation was tested before and after the sulfur containing compounds.

RESULTS. *I. Nicotine action upon the blood pressure of untreated cats after repeated intravenous injection.* In order to ascertain the applicability of the blood pressure raising effect of nicotine as a test procedure before and after the infusion of the sulfur containing compounds, it was necessary first to investigate the con-

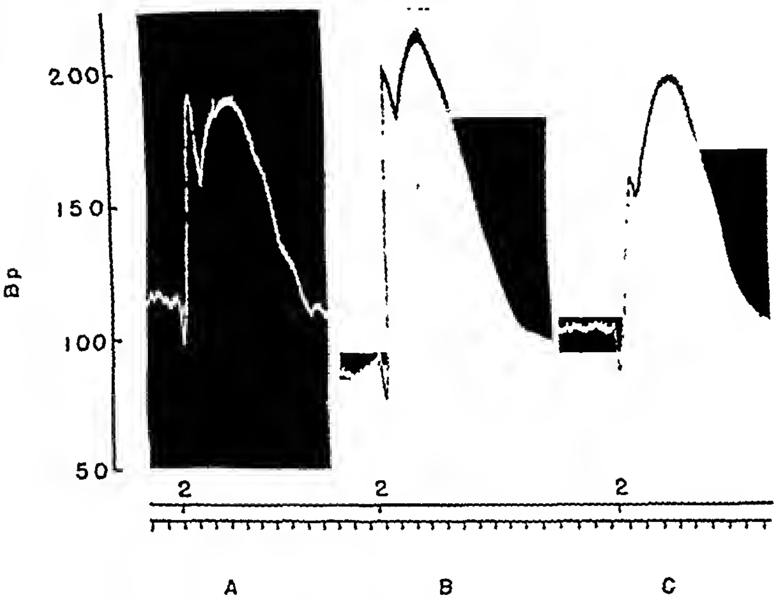

FIG. 1 NICOTINE ACTION UPON BLOOD PRESSURE OF UNTREATED CATS; 10 INTRAVENOUS INJECTIONS OF 1 MGM NICOTINE BASE WERE GIVEN DURING 3 HOURS; THE BLOOD PRESSURE RAISING EFFECT OF NICOTINE REMAINS UNCHANGED

Cat, 2 92 kgm body weight; 30 mgm per kg nembutal; 2 mgm. atropine sulfate Time interval 30 seconds At *2* intravenous injection of 1 mgm. nicotine base 1:1000 *A* 2:07 p.m., 1st; *B* 3·07 p m , 5th; *C* 4:37 p m 10th injection

stancy of the nicotine blood pressure effect after repeated intravenous injections. We injected, therefore, in various experiments 10 to 12 times successively at intervals of 15 minutes, 1 mgm. of nicotine base in a 1:1000 aqueous solution. It was demonstrated in these 3 hour experiments that the response of the blood pressure to intravenous injection of nicotine remained substantially unchanged (fig. 1). It was possible, therefore, to use without hesitation repeated intravenous injections of nicotine in our subsequent experiments for a test of the blood pressure response.

II. Infusion of sodium sulfathiazole in untreated cats. The previous experiments (1, 2) on the isolated gut mentioned initially demonstrated that of all

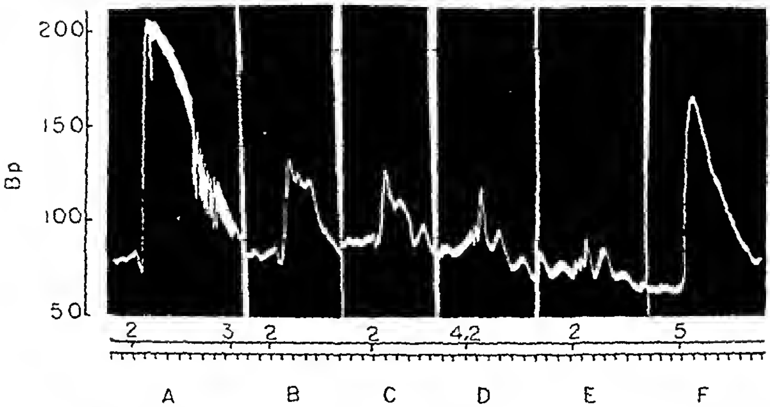

FIG 2 SODIUM SULFATHIAZOLE PREVENTS NICOTINE ACTION UPON BLOOD PRESSURE ADRENALIN ACTION REMAINS UNCHECKED

Cat, 3 65 kgm body weight, 30 mgm per kgm nembutal, 2 mgm atropine sulfate Time interval 30 sec At *2* i v injection of 1 mgm nicotine base 1 1000, at *3* start, at *4* end of infusion of 2% sodium sulfathiazole, at *5* i v injection of 10γ adrenaline 1 1000 *A* 4 15 p m nicotine action before infusion of sodium sulfathiazole, *B* 4 38 p m after infusion of 340 mgm , *C* 4 53 p m after 580 mgm , *D* 5 07 p m after 960 mgm sodium sulfathiazole, *E* 5 13 p m nicotine action, *F* 5 19 p m adrenaline action

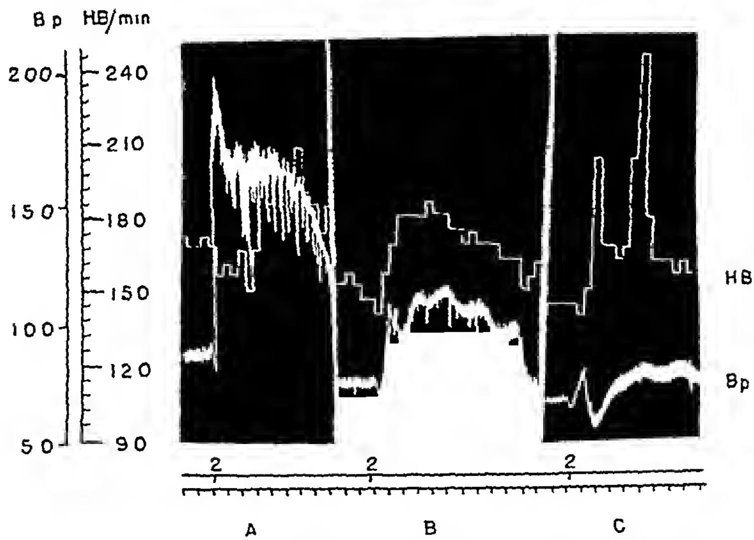

FIG 3 BLOCKING ACTION OF SODIUM SULFATHIAZOLE UPON NICOTINE BLOOD PRESSURE WITHOUT INFLUENCE ON THE HEART RATE

Cat 2 23 kgm body weight, 30 mgm per kgm plus an additional 6 mgm nembutal, 4 5 mgm atropine sulfate Time interval 30 sec At *2* i v injection of 1 mgm nicotine base 1 1000 Recording of heart rate beats per minute *A* 10 47 a m nicotine action before infusion of 1% sodium sulfathiazole 10 57 a m start of infusion *B* 11 32 a m 600 mgm , *C* 11 47 a m 900 mgm sodium sulfathiazole

sulfonamides tested, sodium sulfathiazole had the strongest inhibitory effect with regard to the nicotine spasm of smooth muscle. We therefore used sodium sulfathiazole in the present experiments. First, however, it had to be ascertained that sulfathiazole alone, if infused in considerable amounts, would not influence blood pressure or circulation of normal cats in acute experiments. For this purpose we infused 1–2% solutions of the drug into cats under nembutal anesthesia. It was found, that the infusion of 2.1, 2.4, and 3.0 gm. of sulfathiazole (approximately 1 gm. per kgm. body weight), caused no apparent influence upon the nervous or muscular apparatus of the vascular system. Thus the responsiveness of the peripheral vascular system to adrenaline or acetylcholine remained uninfluenced and the function of the sympathetic ganglia gave no indication of a changed reaction in the sense of a stimulation or inhibition.

III. Nicotine action upon the blood pressure of cats pretreated with sodium sulfathiazole. As was shown above (fig. 1) the adrenaline-like raising of the blood pressure by nicotine can be reproduced as often as desired in the untreated cat. In cats, however, pretreated by intravenous administration of sodium sulfathiazole, the blood pressure raising effect of a given nicotine test dose can be partially or completely suppressed depending upon the amounts of sulfathiazole and nicotine used. This inhibition started at times as early as 8 minutes after infusion of 120 mgm. of sulfathiazole in 2% solution; 100 mgm. were without effect and 300 mgm. resulted in some experiments in a partial, in others in a complete suppression of the nicotine effect upon the blood pressure. In a similar way 500, 600, and 900 mgm. produced an immediate or only slightly delayed decrease or abolition of ganglionic stimulation by nicotine (figs. 2 and 3). This sulfathiazole inhibition or suppression of the nicotine stimulation is not permanent. At times complete inhibition can disappear after 25 minutes and can again be induced by additional sulfathiazole infusion. The relative shortness of the inhibition period can be easily explained by the rapid disappearance of sulfathiazole from the blood stream and its ready elimination from the organism.

This inhibition of the nicotine effect on blood pressure by sulfathiazole is limited to the sympathetic ganglia which stimulate adrenaline secretion and does not extend to the function of the sympathetic heart ganglia. Thus nicotine causes an acceleration of the heart rate quite as if no sulfathiazole had been infused (fig. 3, B.C.). Dixon (3) observed in 1924 that nicotine has positive ino- and chronotropic effects on the isolated heart and Hoffman et al. (4) working in Krayer's laboratory on heart lung preparations of dogs, found recently that nicotine in doses of 1–10 mgm. produces a very marked effect on the dynamic condition of the atropinized heart: increase of rate, decrease of venous pressure and increase of aortic and coronary flow. Our record (fig. 3) shows that despite the blocking of the nicotine effect upon the blood pressure, the action of nicotine on the heart rate (see B and C) and obviously on the other dynamic conditions of the heart remains unaltered.

IV. Nicotine action upon blood pressure of cats pretreated with sodium sulfanilamide, sodium sulfadiazine and sodium sulfapyrazine. Whereas sulfathiazole in the range of 120-300 mgm. regularly produced an inhibition of the nicotine effect

upon the blood pressure, the other sulfonamides tested were much weaker, if at all effective. It was found that a 1% solution of *sodium sulfanilamide* did not influence the nicotine effect even after infusion of 2–3 gm., whereas the subsequent infusion of 300 mgm. of a 2% sodium sulfathiazole solution produced an immediate inhibition of the nicotine effect (fig. 4). *Sodium sulfadiazine*, which showed only partial inhibition of nicotine in the isolated gut, proved also entirely without effect; even after infusion of 3 gm. of a 2% solution and with a blood concentration of 150 mgm.%, the nicotine effect remained entirely intact. The sodium salt of *sulfapyrazine*, which is closely related to sulfadiazine, proved also uncertain in its action; while in one experiment the infusion of 1.5 gm. in a 2%

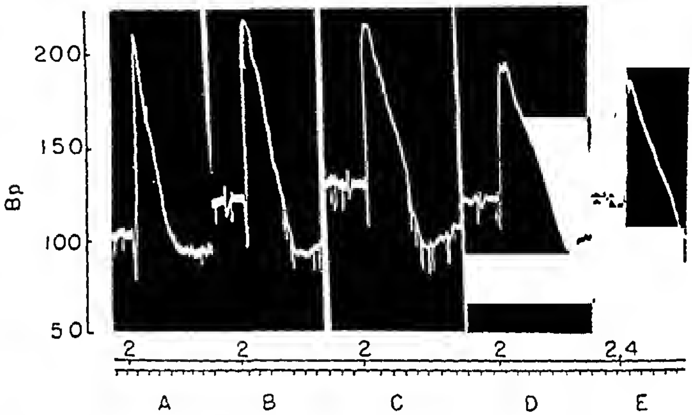

FIG. 4. SODIUM SULFADIAZINE DOES NOT INFLUENCE NICOTINE ACTION UPON THE BLOOD PRESSURE UNLESS IN VERY LARGE DOSES

Cat, 2.98 kgm. body weight; 30 mgm. per kgm. plus 10 mgm. nembutal; 4 mgm. atropine sulfate. Time interval 30 sec. At *2* 0 5 mgm. nicotine base 1:1000. *A* 3:09 p.m. nicotine action before infusion; between *A* and *B* 3:13 p.m. start of infusion of 2% sodium sulfadiazine; *B* 3:39 p m. 500 mgm.; *C* 3:54 p.m. 1000 mgm.; *D* 4:39 p.m. 2200 mgm.; *E* 4:54 p.m. 3000 mgm. sodium sulfadiazine (150 mgm. % in the blood at end of infusion *4*).

solution weakened the nicotine effect, the infusion of 3 gm. in another experiment was without effect.

V. Nicotine action upon blood pressure of cats pretreated with thiamine hydrochloride. Experiments by Unna and Pick (2) proved that *thiamine* as well as *thiazole* inhibit nicotine spasm whereas the pyrimidine part of the thiamine had no such action. It seemed indicated, therefore, to investigate whether pretreatment with thiamine could influence the rise in blood pressure caused by nicotine in the intact animal. We used a 1:1000 solution of crystalline thiamine hydrochloride Merck, which was neutralized with sodium hydroxide before intravenous injection. The inhibitory effect was tested by intravenous injection or infusion of various amounts of thiamine in the range of 2.5 to 30 mgm. per kgm. body weight. For the testing of the blood pressure effect we used nicotine in amounts of 0.1., 0.25, 0.4, and 0.5 mgm. The thiamine inhibition varied in accord-

ance with the dosage of this drug and was especially conspicuous with small amounts of nicotine. In a cat weighing 1.86 kgm. for instance, 5 mgm thiamine hydrochloride produced complete inhibition of the blood pressure raising effect of 0.1 mgm. nicotine base (fig. 5), but only a partial inhibition when tested with 0.25 mgm. of nicotine; the effect of this latter dose could be inhibited only by infusion of 10 mgm. thiamine hydrochloride. In another cat, however (weighing 1.58 kgm.), 5 mgm. of thiamine hydrochloride suppressed the blood pressure raising action of 0.4 mgm. of nicotine completely. In still other experiments with cats of approximately equal weights infusions of 30 to 50 mgm. of thiamine hydrochloride were needed in order to abolish the blood pressure raising effect of 0.5 mgm. nicotine base (fig. 6). The thiamine inhibition, like the sulfathiazole

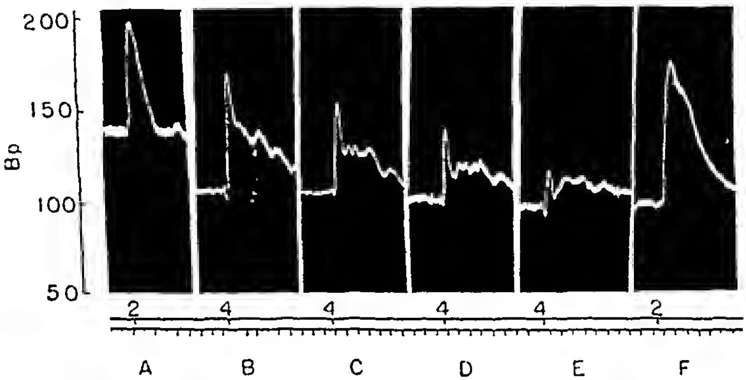

FIG 5 THIAMINE HYDROCHLORIDE IN SMALL DOSES (2 7 MGM PER KGM) PREVENTS NICOTINE ACTION UPON THE BLOOD PRESSURE ADRENALIN ACTION REMAINS UNCHANGED

Cat, 1 86 kgm body weight, 30 mgm per kgm plus 6 mgm nembutal, 2 mgm atropine sulfate. Time interval 30 sec. At *2* 20γ adrenaline; at *4* 0 1 mgm. nicotine base 1:1000 *A* 2:15 p m 20γ adrenalin, *B* 2:42 p m 0 1 mgm nicotine before infusion of thiamine hydrochloride, at 3:11 p m between *B* and C infusion of 5 mgm thiamine HCl in 5 cc., *C* 3:16 p m , *D* 3·31 p m , *E* 3 49 p m , *F* 3·59 p m

inhibition, is transient, however, it lasts longer than when caused by sulfathiazole and can remain for ¾ of an hour to one hour and more.

It should be stressed as shown in extensive experiments of Molitor and Sampson (5) that thiamine even in very large and otherwise fatal amounts (e.g., after intravenous injection of 300 and 350 mgm. per kgm. in rabbits and dogs) leaves the heart and circulation of the untreated animal uninfluenced. Only the subsequent analysis of the activity of the sympathetic synapses with the help of nicotine indicates that thiamine does have a detectable effect on these nerve structures.

VI. *Action of adrenaline, acetylcholine, acetyl-β-methylcholine and carbaminoyl choline upon the blood pressure of cats pretreated with sulfathiazole and thiamine.* Whereas the pretreatment of cats with thiazole containing organic compounds (with sodium sulfathiazole in the range of 120–300 mgm. per kgm. body weight

and with thiamine hydrochloride in amounts of 2.5–30 mgm. per kgm. body weight), causes an inhibition or abolition of the nicotine effect upon the blood pressure, it was demonstrated that the normal effects of adrenaline, acetylcholine, acetyl-β-methylcholine and carbaminoyl choline are in no way changed. This is remarkable in many repects. On the one hand the action of the *free intravenously injected adrenaline* remains unchanged at its site of action on the vessel, on the other hand the liberation of adrenaline from the adrenal medulla by nicotine stimulation of the sympathetic synapses can be either partially or completely blocked by thiazole containing drugs (fig. 5).

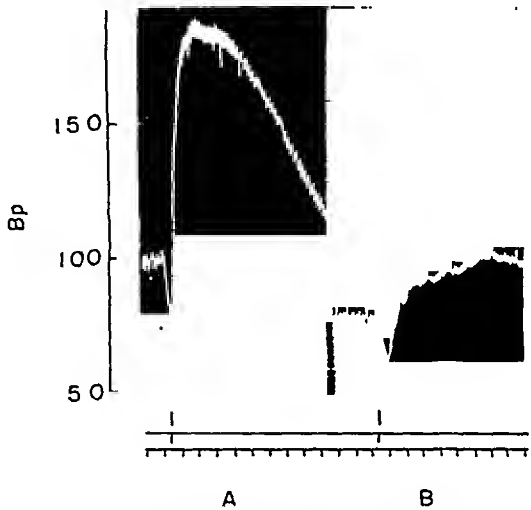

FIG. 6. BLOCKING EFFECT OF THIAMINE HYDROCHLORIDE (16 MGM. PER KGM.) ON NICOTINE ACTION ON THE BLOOD PRESSURE

Cat, 1.85 kgm. body weight; 30 mgm. per kgm. nembutal; 2 mgm. atropine sulfate. Time interval 30 sec. At *I* 0 5 mgm. nicotine base 1:1000. *A* 2:24 p.m. nicotine action before infusion of thiamine; 2:34–2:39 p.m. infusion of 30 mgm. thiamine HCl (0.1% solution); *B* 2:54 p.m. nicotine action 15 min. after end of thiamine infusion; the inhibition remained for an additional 30 min

Acetylcholine is somewhat different in its behavior. Neither its muscarine-like nor its nicotine-like effect are changed essentially. Although in atropinized cats the blood pressure raising effect of acetylcholine, similar to nicotine, involves stimulation of the vasomotor ganglia and increased output of epinephrine (6), it is, in contrast to nicotine, hardly influenced by pretreatment with thiazole containing substances (fig. 7). This indicates that stimulation of sympathetic ganglia by nicotine and by acetylcholine proceeds selectively and independently of each other. Both effects must be based on different chemical processes in the ganglia since the thiazole containing drugs block the nicotine effect while the action of acetylcholine remains undisturbed. This independence of the two rather similar effects on the ganglia agrees with the knowledge that nicotine does

not interfere with the release of acetylcholine in ganglia induced by impulses from cholinergic preganglionic fibres, even though the cells are paralyzed by excessive amounts of cserine or nicotine (7). The independence of the blocked nicotine action and the uninhibited "nicotinic" action of acetylcholine on the sympathetic ganglia brings to mind a similar relationship concerning the blocking of ganglionic transmission by curare: this blocking is not produced by a paralytic action because the involved cells are still capable of responding to other forms of stimuli such as potassium salts and electrical stimulation (8).

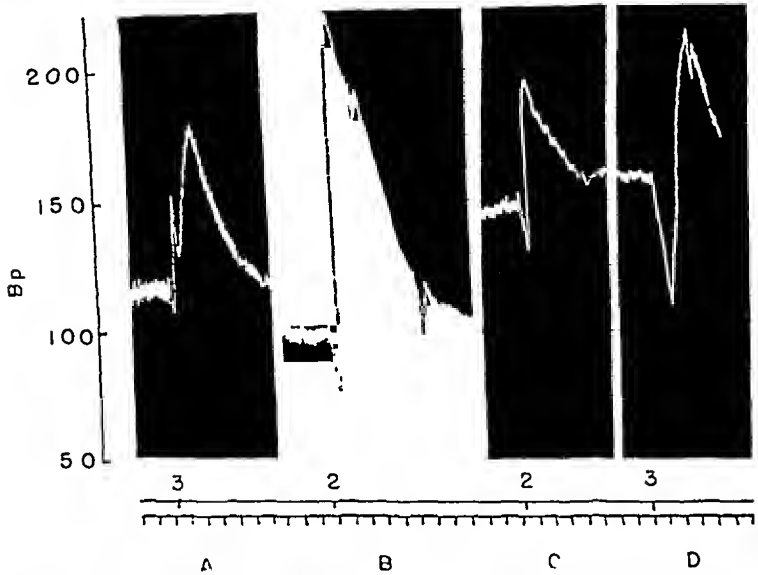

FIG. 7. THIAMINE HYDROCHLORIDE (18 MGM PER KGM) INHIBITS NICOTINE ACTION UPON THE BLOOD PRESSURE, BUT NOT THE ACETYLCHOLINE BLOOD PRESSURE EFFECT

Cat, 2.76 kgm body weight, 30 mgm per kgm nembutal plus 5 mgm, 6 mgm atropine sulfate. Time interval 30 sec. At 2 0.5 mgm nicotine base 1:1000, at 3 3 mgm acetylcholine chlorhydrate. A acetylcholine and B nicotine action before infusion of thiamine, between B and C infusion of 50 mgm thiamine HCl 1:1000. C nicotine action 18 min after end of infusion, D acetylcholine action 5 min later.

VII. Action of the sodium salt of para-aminobenzoic acid on cats pretreated by sulfathiazole and thiamine. It seemed indicated to investigate whether para-aminobenzoic acid which completely reverses the bacteriostatic action of many of the sulfonamides is able to inhibit the thiazole effect upon nicotine or even to abolish an existent blocking effect by subsequent application. For this purpose the sodium salt of para-aminobenzoic acid was injected intravenously before and after nicotine application into cats in amounts of 10–60 mgm. The experiments proved that para-aminobenzoic acid was unable to produce a blockade of ganglia to nicotine and was also unable to abolish an existing blocking effect to nicotine caused by thiazole containing compounds. It can be deduced

from this that the inhibition of the bacteriostatic effect of sulfonamides, one of the longest known competetive receptor effects on bacterial cells has no analogy in nervous cells.

DISCUSSION. The experiments reported herein demonstrate that sulfathiazole and thiamine, in a manner similar to the blocking of the nicotine effect on the isolated gut, are able to inhibit the stimulation of vasomotor synapses and the rise in blood pressure caused by nicotine in the intact animal. As has been demonstrated previously, it was solely the thiazole component of the thiamine which caused the blocking effect on the gut, whereas the pyrimidine component remained ineffective. In perfusion experiments on frogs prepared according to Laewen-Trendelenburg method, Haimovici and Pick (9) found again that only thiazole produced blocking of the nicotine induced vascular contraction, whereas pyrimidine was without effect. In these experiments it was found that of the four sulfonamides tested (sulfanilamide, sulfadiazine, sulfapyrazine and sulfathiazole) only the last named caused blocking of the nicotine action although in a significantly weaker degree than the thiamine; from this we assume that in our nicotine blocking experiments the thiazole group accounts for the blocking action and cannot be replaced in this action by either a pyrimidine or pyrazine group. The fact that sulfathiazole acts only in larger dosages, about 60–150 mgm. per kgm. body weight, whereas thiamine is able to block the nicotine effect upon the blood pressure in as small an amount as 2.5–3.0 mgm. per kgm. body weight and a blood concentration of 2.4 mgm. per cent, is not surprising if we remember how small changes in the molecular structure can influence the various inhibitory effects of antibiotics according to investigations of D. W. Woolley and coworkers and others (10). These relatively small effective amounts of thiamine permit the assumption that in therapeutic application of similar amounts, pharmacological effects on the sympathetic synapses of the human body might be obtained, as observed in the nicotine inhibition on cats. It seems definitely possible that thiamine which is produced according to Muralt (11) in considerable amounts during nervous activity, functions as an important moderator or inhibitor of the overstimulated sympathetic synapses; this might become especially apparent if substances similar to nicotine or other metabolic products which stimulate sympathetic ganglia and thus liberate adrenaline with consequent rise in blood pressure, appear in the circulation. In this sense thiamine and other thiazole compounds related to it may have an important mediatory function for transmission of nervous impulses in the synapses and may secure the enzymatic balance, which is necessary for an undisturbed function of the ganglia. On the other hand, the stimulation of sympathetic synapses by nicotine might be facilitated in the presence of thiamine deficiency in the central and peripheral nervous system.

In view of the impressive blocking of the nicotine effect on the blood pressure by the thiazole containing compounds, thiamine and sulfathiazole, one feels inclined to think of a competitive action between the pyridine group of nicotine on the one hand and the thiazole group of the two inhibitors on the other hand. This competitive action between nicotine and thiamine brings to mind a similar

displacement of thiamine by pyrithiamine in mice with the production of typical signs of thiamine deficiency described by Woolley and White (12). Up to the present time, inactivation by substrate competition was known to occur essentially in antibiotics of plant origin or vitamins. Here, for the first time, a specific nerve poison which leads to vasoconstriction and blood pressure rise is rendered harmless by competitive displacement. Aside from the fact that the blocking of the nicotine action is only temporary, a paralysis of sensitive nerve cells can be ruled out on the basis of the fact that the inhibition of nerve cells is strictly specific for nicotine whereas the same synapses remain fully responsive for other similarly acting posions such as acetylcholine. That the blocking effect of thiazole concerns specifically only the sympathetic ganglia and not the peripheral nerve ends can be easily demonstrated by the observation that drugs which act on the sympathetic or parasympathetic nerve endings show no change in their effectiveness despite complete inhibition of the nicotine action.

It seems justifiable to assume that thiazole containing compounds interfere in some way with enzymatic processes of the cell and thus disturb the nicotine stimulation in or on the synapses, since the nicotine molecule as such is neither destroyed nor altered. Various inhibitory mechanisms of enzymatic processes are well known from the study of the antibacterial actions of the sulfonamides; it can be assumed therefore, that thiazole introduces certain changes in the enzymatic cell function of the sympathetic ganglia which lead to an inhibition of the nicotine action, possibly the site of action is changed to such an extent that the original pharmacological action is abolished.

SUMMARY

1. The intravenous infusion of sodium sulfathiazole (about 60–150 mgm. per kgm. body weight) or of thiamine hydrochloride (about 3–10 mgm. per kgm. body weight), prevents the rise in blood pressure induced by the intravenous injection of 0.1–1.0 mgm. nicotine base in untreated cats.

2. This blocking of the nicotine effect is not caused by a paralysis of the synapses, it lasts 10 minutes to 1 hour or more and is dependent directly in its duration and intensity upon the amount of sulfathiazole or thiamine infused and inversely upon the test dose of nicotine used.

3. The blocking of the nicotine action upon autonomous ganglia is produced essentially by the thiazole group of the sulfathiazole and thiamine, thiazole-free sulfonamides, such as sulfanilamide, sulfadiazine and sulfapyrazine, did not show this effect even in large dosages.

4. The interference with the nicotine effect is probably caused by a transient binding of the thiazole-containing drugs at or inside the sympathetic ganglia as well as at the cells of the adrenal medulla, thus producing a selective displacement of nicotine and its action. It would seem that enzymatic processes in or at these nerve cells are involved.

5. The blockade of the nicotine action which inhibits the rise in blood pressure caused by nicotine, does not seem to influence the vagal and sympathetic ganglia of the heart.

from this that the inhibition of the bacteriostatic effect of sulfonamides, one of the longest known competetive receptor effects on bacterial cells has no analogy in nervous cells.

Discussion. The experiments reported herein demonstrate that sulfathiazole and thiamine, in a manner similar to the blocking of the nicotine effect on the isolated gut, are able to inhibit the stimulation of vasomotor synapses and the rise in blood pressure caused by nicotine in the intact animal. As has been demonstrated previously, it was solely the thiazole component of the thiamine which caused the blocking effect on the gut, whereas the pyrimidine component remained ineffective. In perfusion experiments on frogs prepared according to Laewen-Trendelenburg method, Haimovici and Pick (9) found again that only thiazole produced blocking of the nicotine induced vascular contraction, whereas pyrimidine was without effect. In these experiments it was found that of the four sulfonamides tested (sulfanilamide, sulfadiazine, sulfapyrazine and sulfathiazole) only the last named caused blocking of the nicotine action although in a significantly weaker degree than the thiamine; from this we assume that in our nicotine blocking experiments the thiazole group accounts for the blocking action and cannot be replaced in this action by either a pyrimidine or pyrazine group. The fact that sulfathiazole acts only in larger dosages, about 60–15C mgm. per kgm. body weight, whereas thiamine is able to block the nicotine effect upon the blood pressure in as small an amount as 2.5–3.0 mgm. per kgm. body weight and a blood concentration of 2.4 mgm. per cent, is not surprising if we remember how small changes in the molecular structure can influence the various inhibitory effects of antibiotics according to investigations of D. W. Woolley and coworkers and others (10). These relatively small effective amounts of thiamine permit the assumption that in therapeutic application of similar amounts, pharmacological effects on the sympathetic synapses of the human body might be obtained, as observed in the nicotine inhibition on cats. It seems definitely possible that thiamine which is produced according to Muralt (11) in considerable amounts during nervous activity, functions as an important moderator or inhibitor of the overstimulated sympathetic synapses; this might become especially apparent if substances similar to nicotine or other metabolic products which stimulate sympathetic ganglia and thus liberate adrenaline with consequent rise in blood pressure, appear in the circulation. In this sense thiamine and other thiazole compounds related to it may have an important mediatory function for transmission of nervous impulses in the synapses and may secure the enzymatic balance, which is necessary for an undisturbed function of the ganglia. On the other hand, the stimulation of sympathetic synapses by nicotine might be facilitated in the presence of thiamine deficiency in the central and peripheral nervous system.

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5. The blockade of the nicotine action which inhibits the rise in blood pressure caused by nicotine, does not seem to influence the vagal and sympathetic ganglia of the heart.

6. The nicotine-like blood pressure effect of acetylcholine induced by stimulation of the sympathetic synapses and the adrenal medulla in the atropinized animal, remains unchanged in the presence of a thiazole induced blockade of these same ganglia to nicotine; the effect upon the blood pressure of adrenaline, carbaminoyl choline and acetyl-β-methylcholine likewise remain unchanged.

7. The sodium salt of para-amniobenzoic acid proved incapable of producing or abolishing a blockade of sympathetic ganglia.

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STUDIES ON THE MECHANISM OF ACTION OF SYMPATHOMIMETIC AMINES

III. THE OXIDATION OF TYRAMINE BY RAT LIVER HOMOGENATES

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Since the original description of tyramine oxidase by Bernheim (1), most workers have experienced some degree of uncertainty in the interpretation of the results obtained when the enzyme has been employed. Tyramine oxidase, obtained from livers of various animals including the rat, was reported to catalyze the deamination of tyramine with an oxygen utilization of one atom per mole of substrate in the presence of 0.05 M cyanide at pH 7.3 (2). The same investigator, using guinea pig liver, observed the uptake of four atoms of oxygen per mole of tyramine at pH 5.2, and, in other preparations, the uptake of only two atoms per mole in either alkaline or acid solutions, the difference apparently being due to age of the preparation. Both types of preparation catalyzed the utilization of only one atom of oxygen per mole in the presence of 0.005 M cyanide. Bernheim also was able to demonstrate the formation of p-hydroxyphenylacetic acid when two atoms of oxygen per mole were used, thus corroborating the *in vivo* results of Ewins and Laidlaw (3).

Pugh and Quastel (4) using liver slices rather than the dispersions employed by Bernheim have investigated the oxidation of tyramine and other amines and have reported the oxidation of these compounds in the presence of 0.03 M cyanide with the uptake of one atom of oxygen, and the liberation of one molecule of ammonia, per mole of substrate

Philpot (5), using both liver slices and dispersions at pH 7.4, obtained one equivalent of oxygen uptake with slices, and two equivalents with suspensions, all uptakes above one equivalent being cyanide sensitive It should be mentioned, in view of the results to be reported here, that Philpot observed the formation of both the corresponding aldehyde and acid, whereas Bernheim did not report the isolation of the aldehyde in her first paper on the subject. Pugh and Quastel were able to isolate the corresponding aldehyde when isoamylamine was oxidized but gave no data with respect to p-hydroxyphenylacetaldehyde formation. Thus it would seem that the oxidative reaction does not necessarily cease when the aldehyde has been formed Beyer (15) employed guinea pig liver homogenate as a source of amine oxidase, and was able to obtain reproducible results by employing fresh homogenate and fresh cyanide at all times.

Several investigators (1, 16, 17) have attempted the purification of the enzyme, usually with only moderately good results In view of the marked species variation of occurrence of the enzyme, together with its difficulty of purification, one

may postulate, as Alles and Heegaard (16) have suggested, that "amine oxidase" is in reality a mixture of several enzymes acting together or in sequence.

After consideration of these results, it seemed to us of interest to investigate the mechanism of oxidation of tyramine, employing the well known effect of dilution by homogenization, together with the addition of various substances suspected of acting catalytically. In order to limit variability of results as much as possible only rat livers were used, and the reaction was carried out in an alkaline medium.

METHODS. Mature white rats of Wistar descent were decapitated and liver homogenates in distilled water were prepared immediately. Oxygen uptake was determined in Summerson constant volume differential manometers.

Cytochrome C was prepared by the method of Keilin and Hartree (6) and was stored in the lyophilized (7) state. Coenzyme I was prepared from Baker's yeast by the method of Williamson and Green (8). Dimethylglycine was prepared by the method of Michaelis and Schubert (9).[1]

A 10% liver homogenate was allowed to catalyze the oxidation of the compound in an atmosphere of air at 37°, the pH being maintained at 8.8 by dimethylglycine buffer. Various substances were added, and the effect on oxygen uptake noted. Since the amount of active enzyme in liver apparently is low, it was not found practical to dilute further than 10%, even though the effect of addition of catalytic substances could not be demonstrated as vividly as would have been possible if the tissue could have been used in a more dilute state.

The following substances were added, and their effect on tyramine oxidation recorded: cytochrome C, coenzyme I, aluminum ions, calcium ions, magnesium ions, pyruvate, glutamate, succinate, methylene blue, cocarboxylase, glutathione, adenine, adenosine, adenylic acid, adenosine triphosphate, and sodium chloride. p-Hydroxyphenylacetic acid was found to be strongly inhibitory to both tyramine oxidation and tissue respiration. Of these compounds, cytochrome C, coenzyme I, aluminum and calcium ions, pyruvate, glutamate, and methylene blue were found to be stimulatory. The other substances mentioned proved either to have no effect or to be slightly inhibitory.

After considerable experimentation the following set-up was found to give the most consistent results. (Tyramine was placed in the right hand vessel of the Summerson manometer in each case, the left hand vessel containing all of the other components as a control):

	Left Vessel ccm.	*Right Vessel* ccm.
10% homogenate	0.5	0.5
Cytochrome C, 4×10^{-4}M	0.3	0.3
Coenzyme I, 0.75%	0.2	0.2
Nicotinamide M/10	0.1	0.1
$AlCl_3$ 0.004 M	0.1	0 1
$CaCl_2$ 0.004 M	0.1	0.1
Pyruvate M/1 pH 6.0	0.3	0.3
Methylene blue 0.001%	0.1	0.1
Dimethyl glycine buffer M/1, pH 8.8	1.1	0.9
Tyramine HCl, 0.018 M		0.2*
KOH, 20% in center well	0.2	0.2

* In side bulb.

[1] We are indebted to Dr. E. M. Schultz of the Dept. of Organic Chemistry, of this Division, for the preparation of this compound, and for the purification of the pyruvic acid used.

RESULTS AND DISCUSSION. Before discussing the results obtained, further comment should be made regarding the addition of some of the above substances.

Nicotinamide, added as an inhibitor of coenzyme I nucleotidase, produced troublesome inhibition of tyramine oxidation at times. This has previously been noted by Utter and coworkers (10), and is probably dependent on the initial concentration of tissue cozymase.

In early experiments sodium glutamate, added to both vessels as a substrate, was found to accelerate the rate of oxidation of tyramine. In these experiments analysis for α-ketoglutarate showed the presence of this compound, thus suggesting that the glutamate might be active because of its oxidative deamination to the keto acid. We reasoned that if this were true, pyruvate also should be active. This was indeed the case and pyruvate rather than glutamate was used in subsequent experiments. Pyruvate usually was added to make a final concentration of 0.1 M.

Methylene blue in high concentrations was found to inhibit the oxidation of tyramine and to stimulate in low concentrations. When used, it was employed in a final concentration of 0.003 M. It was found to reduce the number of sluggish and rather inactive preparations, which we believed possibly due to deficiency, dilution, or destruction of cytochrome oxidase. Methylene blue was not used in the experiments involving cyanide described below.

Figure 1 demonstrates the stimulatory effect of cytochrome C, coenzyme I, pyruvate, and methylene blue. In this figure, 40 c.mm. of oxygen uptake is equivalent to one atom. One may see here that an uptake of two and one-half atoms of oxygen per mole is recorded. However, this is not always the case, and sometimes an uptake of three atoms per mole was noted.

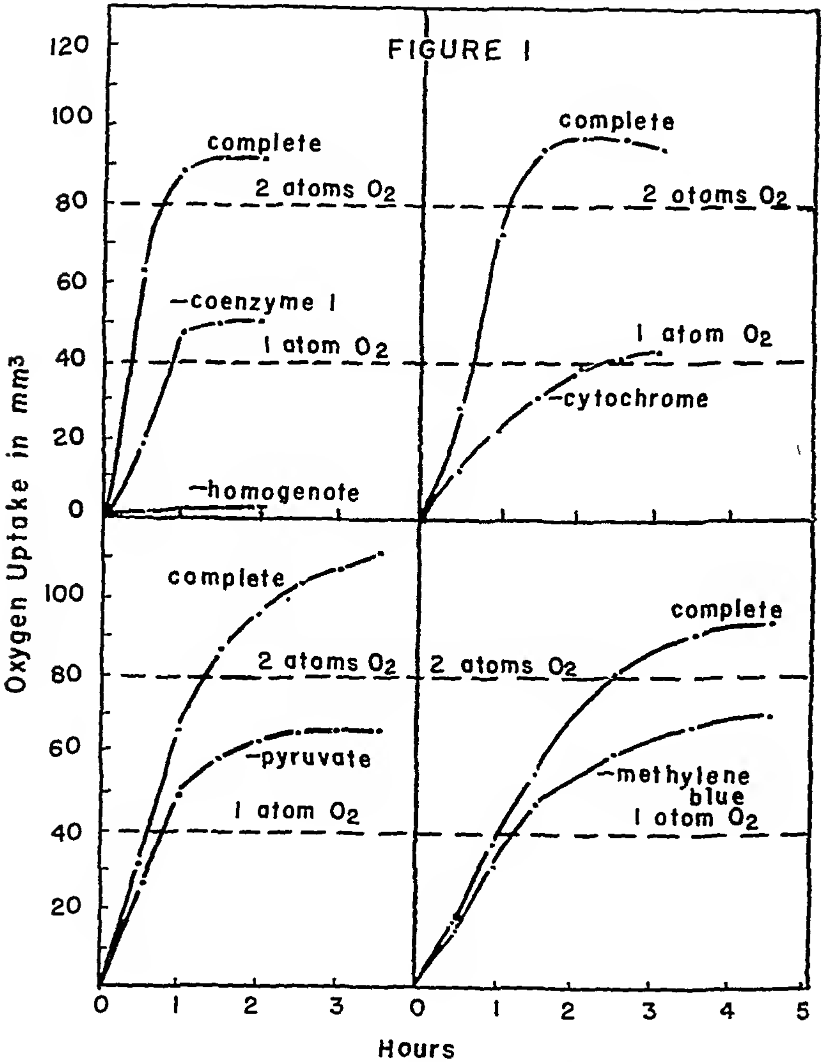
From the foregoing experiments it would appear that we probably were dealing with a dehydrogenase, since coenzyme I was stimulatory, and with the cytochrome-cytochrome oxidase system as a possible end path of oxidation. The function of pyruvate seems somewhat in doubt but more is to be said on this subject later. Since we were able to oxidize N-methyl-phenethylamine with oxygen uptakes approaching two atoms per mole in this system, it seems improbable that the bulk of this oxidation was concerned with the ring of tyramine. p-Hydroxyphenylacetic acid would seem to be definitely etablished as the end product when two atoms of oxygen are used, in view of the work of Bernheim and of Philpot already quoted. Table 1 illustrates a reaction scheme that we postulate to explain the experimental observations.

Reactions I to III involve the uptake of two atoms of oxygen per mole of substrate, and require the hypothesis that pyruvic acid is the hydrogen acceptor.[2] In order to ascertain the likelihood of this being true experiments involving varying concentrations of cyanide[3] and of pyruvic acid were undertaken. Theoretically if the cytochrome-cytochrome oxidase system is the end path of the hydrogen transport, and if pyruvate is a carrier, then low concentrations of cyanide

[2] If H_2O_2 is formed in the oxidation of lactate, tissue catalase should destroy it, making the net uptake one equivalent.

[3] The precautions of Krebs (14) were used when cyanide was added.

should inhibit cytochrome oxidase, but should not be sufficient to cause inactivation of all of the pyruvate by cyanhydrin formation. This would result in an oxygen uptake of one atom per mole of tyramine, since sufficient pyruvate would



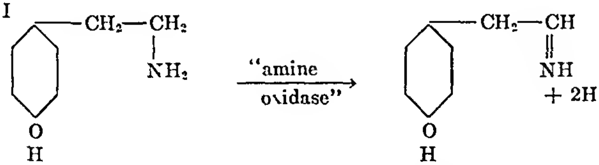
be present to take up all of the hydrogen atoms released in the conversion of tyramine to the aldehyde, and the uptake due to the subsequent oxidation of the aldehyde to the acid would be recorded. Conversely, if no cyanide were present

both this atom of oxygen and that seen from oxidation of the lactate formed would be recorded, since cytochrome oxidase would be intact in this case.

Also, if sufficiently high concentrations of cyanide were used, not only would cytochrome oxidase be inactivated, but the pyruvic acid (in the tissues or added) would be inactivated and reaction I could not occur. If reaction I could not take place, neither could reaction II, and no oxygen uptake should have been recorded Figure 2 illustrates an experiment in which the pyruvate concentration was fixed at M/10 and the cyanide concentration varied from M/10 to

TABLE I

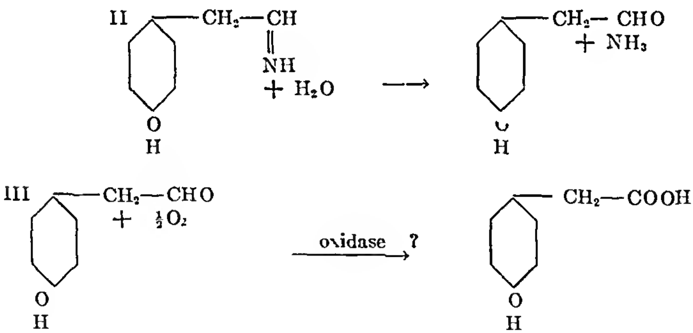
I



$$CH_3COCOOH + 2H \longrightarrow CH_3CHOHCOOH$$

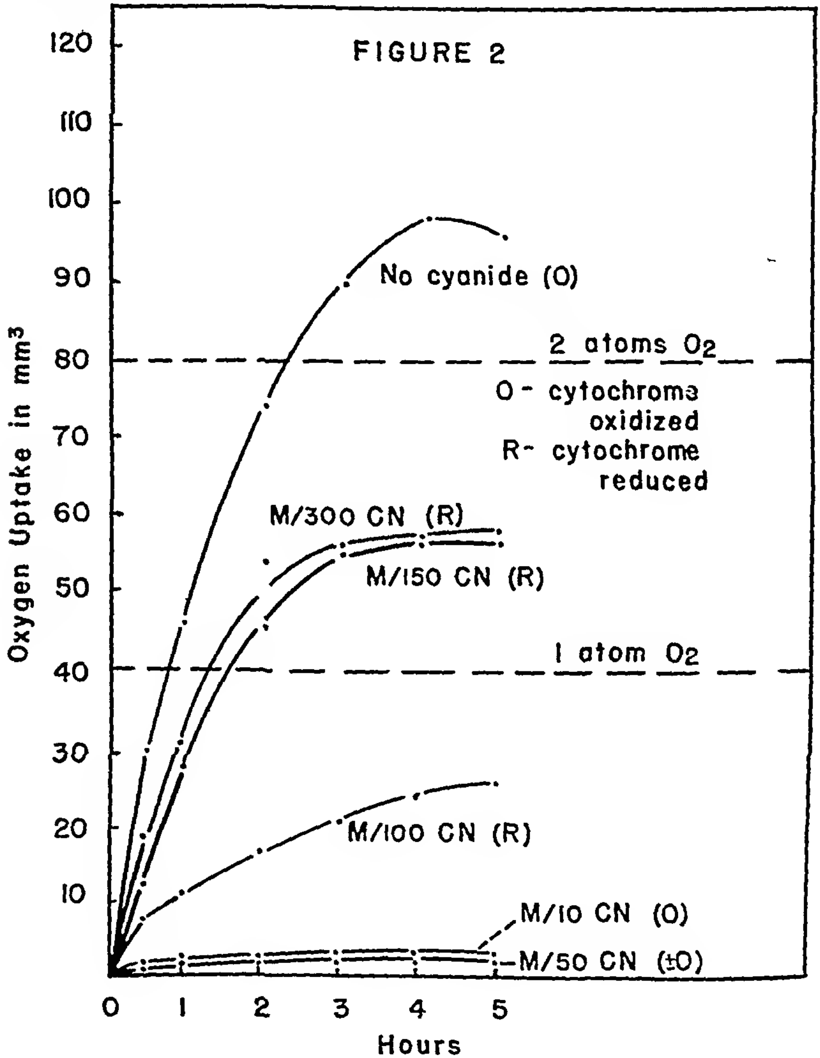
$$\left[CH_3CHOHCOOH + \tfrac{1}{2}O_2 \xrightarrow[\text{dehydrogenase and cytochrome system}]{\text{lactic}} CH_3COCOOH + H_2O \right]$$

II



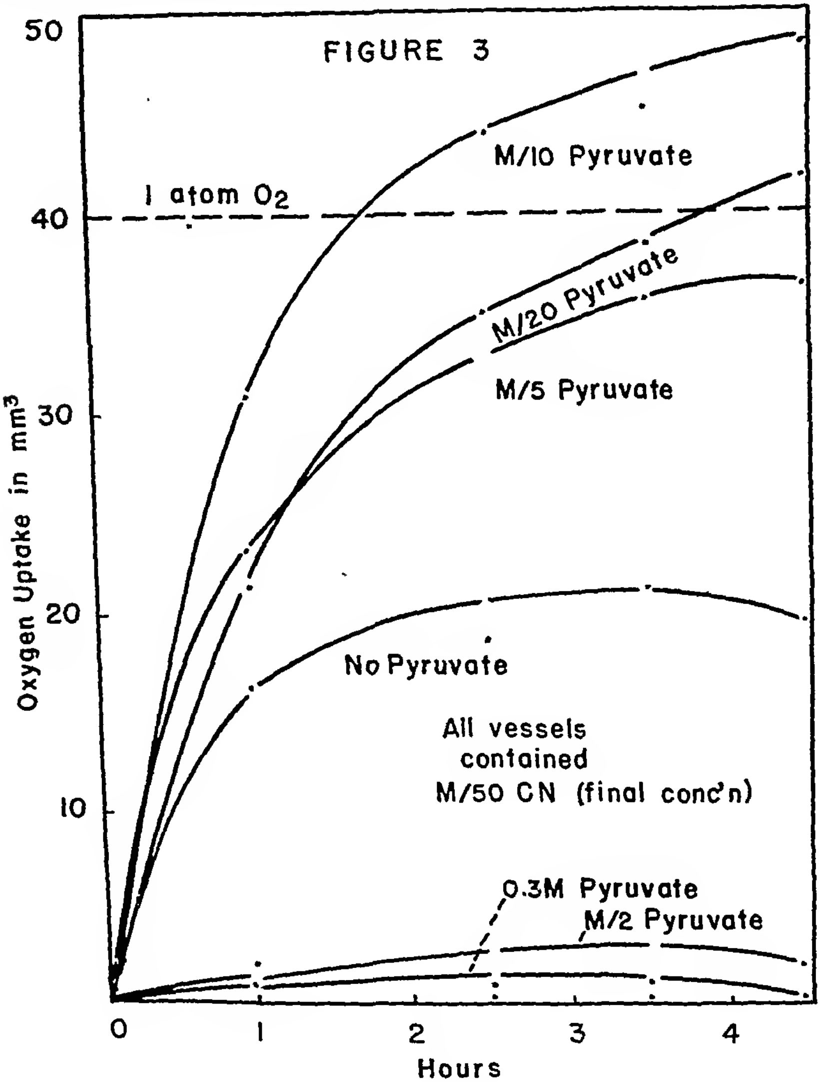
M/300. One may observe that strong cyanide concentrations inhibited all oxygen uptake, whereas dilute cyanide concentrations allowed an uptake of one atom per mole. It should be noted that we obtained an uptake of slightly more than one atom per mole in this case (1 atom ≈ 40 c mm) This "extra" oxygen uptake may be explained on the basis of a bypass around the cytochrome system, or by the supposition that at these low rates of oxygen uptake considerable amounts of cytochrome oxidase may be inactivated and still leave enough active enzyme to serve as carrier since the oxidase is probably not saturated with substrate (11) In any case, this extra uptake of oxygen has never amounted to more than ½ atom per mole.

In order to determine whether or not the failure of oxygen uptake with high concentrations of cyanide could have been due to inhibition of some component of the system other than the pyruvic acid, another experiment was set up, fixing



the concentration of cyanide at M/50 (final) and increasing the pyruvate concentration. In the reaction between cyanide and pyruvate, the cyanide is bound as a cyanhydrin and theoretically it should be possible to add enough pyruvate to leave an excess when the reaction has been completed. Here one runs the risk,

of course, of inhibition of lactic dehydrogenase by pyruvic acid. Figure 3 illustrates such an experiment. In vessels without added pyruvate only the "extra" ½ atom per mole of uptake was observed, whereas addition of pyruvate in excess



allowed the reaction to proceed to the extent of one atom per mole. Still higher concentrations of pyruvate slowed the reaction as would be predicted if the lactic enzyme were inhibited. The uptake did not proceed to two atoms per mole,

probably because sufficient cyanide to inactivate cytochrome oxidase still was present.

We do not believe that the findings of Potter (18), that cyanide in high concentration combines with cytochrome C, are applicable here, since our cytochrome concentrations were much higher than his, and since excess pyruvate negated the inhibition.

Thus we believe that an uptake of one atom of oxygen per mole can be explained by the action of a dehydrogenase with pyruvic (or other keto acid) acting as hydrogen acceptor, with oxidation of the resulting lactic acid in the presence of the lactic enzyme, cytochrome and cytochrome oxidase being the end path. Such systems have been described previously by Green and co-workers (12), who were able to couple xanthine oxidase and hypoxanthine to lactic dehydrogenase and pyruvate both by means of naturally occurring carriers and also by oxidation-reduction indicators. We have not been able to determine the coupling carrier in the oxidation of tyramine but the fact that methylene blue stimulates the system in the absence of cyanide suggests that a natural carrier is present in low concentration.

The oxidation of the aldehyde to the acid possibly may be explained as due to the activity of liver xanthine-aldehyde oxidase, but this enzyme is relatively cyanide sensitive, and one would have to assume that the reaction takes place before complete inactivation of the enzyme. This may be possible, since Dixon (13) states that the cyanide destruction of xanthine-aldehyde oxidase is lessened if enzyme and cyanide are not allowed to incubate before the addition of the substrate. In our case the homogenate was added immediately before equilibration. Philpot (5), by adding uric acid, known to inhibit xanthine-aldehyde oxidase, was able to reduce the oxidation of tyramine by fifty per cent. We wish to emphasize that our consideration of the possible function of xanthine-aldehyde oxidase in this reaction is purely speculative.

It should be mentioned that, although xanthine-aldehyde oxidase commonly is believed to function as a dehydrogenase after hydration of the aldehyde, apparently neither pyruvate nor the cytochrome system is necessary for transfer of hydrogen atoms to oxygen.

One obtains additional corroboration of the theory postulated above by visual observation of the amount of reduction of cytochrome C in the vessels after the reaction has been completed as illustrated in figure 2, those vessels containing high concentrations of cyanide showed no reduction of cytochrome, demonstrating the blocking of the mechanism of cytochrome C reduction, whereas vessels containing dilute cyanide showed reduced cytochrome, indicating that cytochrome oxidase was not available for its oxidation, but that the reducing mechanisms remained intact.

In addition to the oxygen uptakes discussed above, a third atom of oxygen per mole remains to be explained. It has been noted previously that most often only $2\frac{1}{2}$ atoms rather than three atoms, have been recorded. Since it seems improbable that the side chain of tyramine would be oxidized further than to the acid, one naturally would think of the phenol ring as the most likely site of oxidation.

The satisfactory explanation of oxygen uptakes greater than two atoms per mole under these conditions with tyramine as substrate awaits further investigation. Whatever the reaction, its inhibition by the products of oxidation is quite possible since we have noticed a markedly inhibitory effect on tyramine oxidation if p-hydroxyphenylacetic acid were added. This probably would explain why two and one-half rather than three atoms of oxygen uptake usually have been noted.

It would seem that, if our hypothesis be valid, tyramine should be oxidized anaerobically to the aldehyde. When this oxidation was investigated by the Thunberg technique, it was found that the oxidation of natural substrates in liver was so rapid that any dehydrogenation of tyramine could not be seen. The determination of ammonia after incubation of the reaction mixture in an atmosphere of nitrogen likewise failed to reveal evidence of tyramine oxidation. This may mean that the hydrogen acceptors are blocked by oxidation of natural substrates in this case also, or that we are in error as to the course of tyramine oxidation. The final elucidation awaits purification of the enzymes involved.

Thus it would seem, if the above hypothesis be true, that "amine oxidase" may be considered to be a mixture of several enzymes—an "amine dehydrogenase", lactic dehydrogenase, an enzyme capable of oxidizing p-hydroxyphenylacetaldehyde to the acid, and the cytochrome-cytochrome oxidase system. This view may help to explain the difficulties of purification, and the species differences noted by other workers.

Further work is planned along the lines of purifying the components of the systems involved, in the hope that more information may be obtained with regard to the amine oxidase, with the ultimate objective of purification of the dehydrogenase.

SUMMARY

The oxidation of tyramine by rat liver homogenates *in vitro* is stimulated by cytochrome C, coenzyme I, α-keto acids, methylene blue, and aluminum and calcium ions. A possible mechanism of reaction involving a coupled reaction between a dehydrogenase ("amine oxidase") with tyramine as substrate and the lactic dehydrogenase in the presence of pyruvate, the cytochrome-cytochrome oxidase system serving as final path to oxygen, is described. This reaction is followed by oxidation of the p-hydroxyphenylacetaldehyde to p-hydroxyphenylacetic acid, possibly catalyzed by xanthine-aldehyde oxidase. Oxidation of the ring of tyramine also is suggested.

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AN EVALUATION OF THE RHODAMINE B METHOD FOR THE DETERMINATION OF ANTIMONY[1]

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INTRODUCTION

In the course of an investigation of therapeutic agents in experimental leishmaniasis, two methods for the quantitative determination of antimony in biological material have been tried in this laboratory: polarography (1), and the colorimetric method utilizing rhodamine B (2, 3). Of these two methods the latter has, in our experience, provided the best combination of rapidity, specificity, and accuracy.

The experiments here reported were made to evaluate critically the reproducibility of antimony determinations by the rhodamine B method, and to determine the extent of the recovery of antimony when added to tissues in the form of several therapeutically important organic antimonials. The results have been analyzed by standard statistical procedures.

PROCEDURE. The procedure used is essentially that of Maren (3), with certain modifications which in our experience led to greater reproducibility.

1. Tissue, plasma, or urine is placed in 50 ml. Erlenmeyer flasks; 3 ml. of concentrated sulfuric acid, 5 ml. concentrated nitric acid are added, as well as 4 or 5 glass beads to minimize bumping.

2. The samples are digested on an electric hot plate which is capable of providing graded heat increases. The digestion is carried out on "low" (i.e. about 180°C.) until charring occurs, or in the absence of charring, until the evolution of SO_3 fumes. If, during this stage, charring does occur, the flasks are removed from the hot plate, and, after cooling, an additional 5 ml. of concentrated nitric acid are added and the procedure repeated. As frequently as charring occurs, the addition of 5 ml. of concentrated nitric acid is repeated until a colorless solution is present at the stage of SO_3 evolution. At this time, the heat is advanced rapidly to "medium" (about 260°C.) and then to "high" (about 340°C.), care being taken to avoid violent bumping. Not infrequently charring will occur when the heat is increased and this necessitates yet another nitric acid treatment and repetition of the procedure described above Although the volume of nitric acid required in the digestion rarely exceeds 20 ml., quantities of nitric acid up to 50 ml. have been used in a control blank without introducing a detectable reading after color development. When the digestion has been carried to the stage where the heat is on "high" and the solutions are colorless, the heating must be continued until all traces of nitric acid and its decomposition products have been removed. In our experience, a period of 20 minutes has been found to be adequate to accomplish this. We believe that this is a critical factor in obtaining reproducible values. Control experiments have shown that the addition of as little as 0.02 ml. of con-

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

centrated nitric acid to an unheated sample containing 6.0 micrograms of antimony results in as much as a 50 per cent reduction in the final color developed.

The analysis may be interrupted for an indefinite period at any stage up to this point but must be continued without delay after the next stage is begun.

3. Three ml. of freshly prepared 1 per cent sodium sulfite are added, and the solution heated again until dense white fumes of SO_3 are produced, and for 5-15 minutes thereafter.

4. The solution is cooled, 2.5 ml. of water are added and the flasks placed in an ice-water bath until the solution is at room temperature or below.

5. 2.5 ml. of concentrated hydrochloric acid are added. It is essential that the solution be at room temperature or below during the addition of the HCl. Higher temperatures lead to erratic color development. Experience has shown that it is very difficult to cool the solution adequately when the hydrochloric acid is added directly to the concentrated sulfuric acid solution obtained from step 3. Accordingly, the dilution step which produces most of the heat is carried out separately from the hydrochloric acid addition.

6. One drop of ceric bisulfate[2] ($Ce(HSO_4)_4$) solution is added, the contents of the flask are well mixed, and the solution is allowed to stand in the ice-water bath for 2 minutes.

Under the conditions here employed, one drop of ceric bisulfate solution yields an excess as indicated by the yellow color. More than 4 drops (0.1 ml.) may produce a reduction in the final color with rhodamine B.

7. Eight ml. of 3 N phosphoric acid solution are added and the solution is well mixed.

8. Five ml. of 0.02 per cent rhodamine B solution are then added with *vigorous* mixing. It is essential that thorough, continuous mixing of the reagents occurs; otherwise the intensity of color developed from a given quantity of antimony may be variable. The solution is returned to the ice-water bath until it is below room temperature.

9. The aqueous mixture is transferred as completely as possible to a 125 ml. separatory funnel, and exactly 10 ml. of benzene are added. The contents of the separatory funnel are shaken 400 times and the layers allowed to separate. The aqueous layer is drawn off and discarded; the benzene solution is transferred to a 15 ml. centrifuge tube and centrifuged lightly in a stoppered tube to remove small water droplets which, if dispersed in the benzene, tend to scatter light and give a falsely high value for the spectrophotometric reading.

10. The benzene solution containing the colored antimony-rhodamine B complex is poured into absorption cells of 1.0 cm. light path and the absorption, as $\log_{10} \frac{I_o}{I}$ at 565 mμ, is read directly off the scale of the Beckman photoelectric spectrophotometer.[3]

Results. *Standard antimony calibration curve.* Antimony trioxide, Sb_2O_3, was used for the standardization of the method. 119.7 mgm. of Sb_2O_3 were dissolved in 1 l. of 1 N HCl to give 100 micrograms of antimony per ml. This standard solution was further diluted, as required, with 1 N HCl to give a working solution containing 10.0 micrograms of Sb per ml. In every instance the determination was carried through exactly as described in Procedure above. The results of this part of the study are summarized in table 1 and figure 1. Table 1 presents the number of observations made at each amount of antimony, the mean density reading obtained on the Beckman spectrophotometer, and the standard deviation of the readings at each amount of antimony. The size of this standard deviation indicates the reproducibility of the technique. From an

[2] One gram $Ce(HSO_4)_4$ suspended in 100 ml. water plus one ml. of concentrated sulfuric acid to effect clear solution.

[3] The value $\log_{10} \frac{I_o}{I}$ is usually referred to in the chemical literature (4) as extinction, E. It is also referred to as "optical density".

inspection of the individual standard deviations it can be seen that there appears to be no systematic increase. In fact the 6 values do not differ significantly from each other since their variation from the average standard deviation of 0.024 could easily be due to chance as determined by a standard statistical pro-procedure, the Chi Square Test. This is fortunate since for ease of further analysis it will be convenient to work with a single value for the standard deviation i.e. 0.024.

Figure 1 presents the mean Beckman readings of table 1 plotted against the corresponding amounts of antimony from 2.0 to 15.0 micrograms. The line was obtained by the method of least squares taking the antimony values as fixed so that the errors in the positions of the points is due to errors of technique in determining the Beckman value. The equation of the line is $y = -0.0435 + 0.0653\,x$ where y is the density reading obtained from the Beckman spectrophotometer and x is the micrograms of antimony. It is to be noted that the observed mean density readings did not in every instance fall on the theoretical

TABLE 1

Average Beckman density readings with known amounts of antimony

ANTIMONY	NUMBER OF OBSERVATIONS	MEAN BECKMAN READING	S D *
micrograms			
2.0	14	0.091	0.019
4.0	14	0.212	0.018
6.0	13	0.320	0.028
8.0	13	0.507	0 021
10.0	13	0.622	0.033
15.0	14	0 925	0.021

$$^*\text{S D.} = \sqrt{\frac{\text{Sum of squared deviations from mean}}{\text{No of readings} - 1}}$$

straight line which relates the Beckman reading to the amount of antimony. This is particularly true at 6.0 and 8.0 micrograms of Sb. However, we have no evidence to indicate that a more complex description of the observations is justified since the remaining points do not deviate significantly from a straight line nor do the observations at 6.0 and 8.0 micrograms show a consistent deviation from the curve.

In order to evaluate the error of the method in terms of the reproducibility of the values given by the line it is necessary to increase the S.D. of 0.024 because of the fact that the averages deviate from the calibration curve. In fact, the square of their deviation from the calibration curve is 0.00031 on the average, so the adjusted S.D. is $\sqrt{(0.024)^2 + 0.00031} = 0.029$. This adjusted S.D. describes both the error in the precision of the method and the error introduced by the fact that the average observations do not fall precisely on the calibration curve. The light lines which parallel the calibration curve in figure 1 are 1 standard deviation (0.029) above and below the line respectively. A line drawn parallel to the abscissa of the graph and joining the 2 standard deviation lines

enables one to read directly the variation of the method in terms of amounts of antimony. Thus it may be seen that at any point on the calibration curve 2 standard deviations are equal to 0.8 micrograms of antimony. This means that the maximum error of the method in the range of 2.0 to 15.0 micrograms of antimony is ± 1.0 micrograms Sb (i.e. 2.5 standard deviation units); in fact, 95 per cent of the time the error will be no larger than ± 0.8 micrograms Sb, and 68 per cent of the determinations will fall within ± 0.4 micrograms of the correct value.

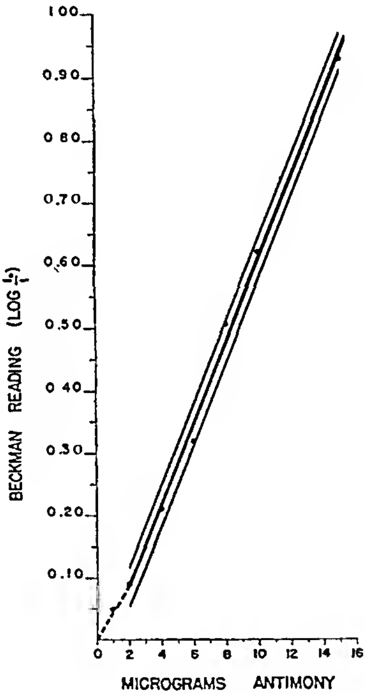

FIG. 1. STANDARD ANTIMONY CALIBRATION CURVE
The light lines parallel to the heavy calibration line represent ±1 standard deviation. See the text for a discussion of their significance.

The standard errors of the intercept (−0.0435) and of the slope (0.0653) are so small that the position of the line is highly stable. It also follows from this that the S.D. (0.4 micrograms) is accurate.

Another feature of the calibration curve that is to be noted is that it is not a rational curve since it does not pass through the zero point. The value −0.0435 differs very significantly from zero. We have no evidence to explain our observation that between 0.0 and 2.0 micrograms of antimony the density readings do not bear the same proportionality to the amount of antimony as from 2.0 to 15.0 micrograms antimony. We have not assessed the precision error from 0.0–2.0

micrograms and we have merely drawn the best free hand line in this range utilizing the available evidence at 1.0 microgram. Wherever possible, in experimental determinations, an attempt will be made to work with amounts of antimony greater than 2.0 micrograms.

From figure 1, the value of $\log \frac{I_0}{I}$ at 565 mμ with 10 micrograms of antimony is 0.622 for a 1 cm. light path in the Beckman spectrophotometer. From figure 1 of Maren's paper (3) the value of $\log \frac{I_0}{I}$ at 565 mμ with 10 micrograms of antimony is about 0.5 for a 1.9 cm. light path in the Coleman No. 11 spectrophotometer; this would mean a value of about 0.26 for a 1 cm. light path. This apparent discrepancy between the present data and that of Maren arises from the difference in characteristics between the Beckman and the Coleman spectrophotometers.

The absorption spectrum of the antimony-rhodamine B complex in benzene, as measured on the same solution (11.2 micrograms antimony) by the two instruments, is given in figure 2. It will be seen that the Beckman, because of the fact that it employs a narrower slit than the Coleman, gives a much higher value of $\log \frac{I_0}{I}$, that is a greater sensitivity at 565 mμ per microgram of antimony. Thus, at 565 mμ our values for the Beckman and Coleman are, respectively, 0.690 and 0.296 for 11.2 micrograms antimony, or 0.62 and 0.26 for 10 micrograms of antimony. Thus, our value 0.26 with the Coleman checks exactly the Maren value of 0.26 per 1 cm. cell.

One further implication of the data of figure 2 should be stressed. The steepness of the absorption curve makes it advisable to leave the wavelength scale set at 565 during the entire series of readings based on a given calibration curve, as relatively slight differences in setting may change the slope of the calibration curve significantly.

Recovery of antimony from tissue. There were two problems presented in this section of the work. First, it was necessary to determine whether the presence of tissue altered, in any way, the intensity of adsorption at 565 mμ exhibited by a given amount of antimony in the rhodamine B complex, and, second, it was necessary to determine whether the chemical state of the antimony, i.e., as inorganic antimony or as chemically bound antimony in the form of organic antimonials affected the final results. It was decided to investigate the recovery from tissues of the antimony of 4 therapeutically important organic antimony compounds: potassium antimony tartrate (tartar emetic), lithium antimony thiomalate[4] (Anthiomaline), diethylamino salt of sodium antimony gluconate[5] (Stibanose), and diethylamino p-aminophenylstibonate[6] (Neostibosan). Suitable aqueous dilutions of these drugs were made either on the basis of the chemical

[4] Supplied by Merck and Co., Lot No. 41765; Manufacturers analysis Sb = 1.0%.

[5] Supplied by Winthrop Chemical Co., Lot. No 421A126; Manufacturers analysis Sb = 28.6%.

[6] Supplied by Winthrop Chemical Co., Lot No 377AJ; Manufacturers analysis Sb = 40–42%.

formula or on information supplied to us by the manufacturer so that the concentration of Sb was theoretically equal to 10.0 micrograms per ml. Known amounts of drug antimony were added to samples of normal hamster liver. The liver was chosen as the standard tissue to assay the success of antimony recovery since in our preliminary experiments and in the literature (5) it has been found that the liver is the chief organ for the deposition of antimony. Quantities of liver ranging from 200–600 mgm. were used. For each drug, determinations were made at 2.0, 8.0 and 15.0 micrograms of antimony. The control series consisted

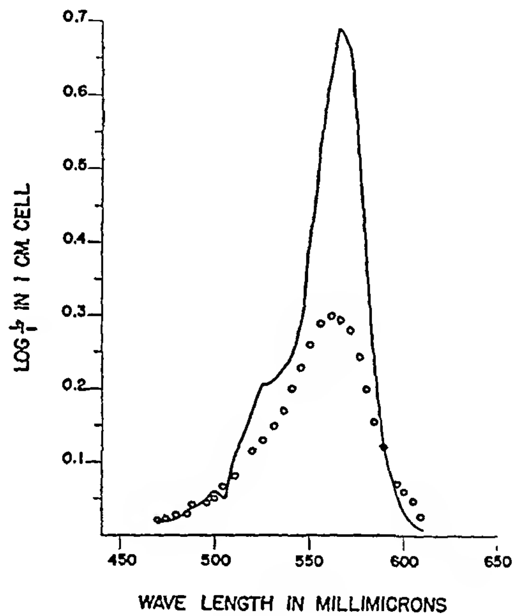

FIG. 2. VALUES OF LOG $\frac{I_0}{I}$ OBTAINED UPON THE SAME 10 ML. OF BENZENE SOLUTION CONTAINING THE AMOUNT OF ANTIMONY-RHODAMINE B COMPLEX FORMED FROM 11.2 MICROGRAMS OF ANTIMONY UNDER THE CONDITIONS DESCRIBED IN THE TEXT

The measurements designated —— and ∘ O were made upon the Beckman and Coleman spectrophotometers, respectively.

merely of the aqueous solutions containing the particular antimonial. Five control and five tissue determinations were made at each antimony value. Table 2 summarizes the results of these experiments. Together with the drug and the amount of antimony, it presents the mean of the five density readings obtained with the Beckman spectrophotometer, the standard deviation of the five readings and Student's t value for the difference between the control and tissue mean. This latter number was computed in each instance in order to test the significance of the difference between the mean density reading of the "control" and of the "tissue" samples. In every instance except 1 there is no significant difference

between the control and liver sample. At 2.0 micrograms of tartar emetic antimony there is a statistically significant difference between the mean values of the control and the liver samples. This was largely due to the fact that in this particular experiment the variation in the determination of both groups happened to be very much less than in other experiments. Because of this the difference between the means, though no greater than in many of the other comparisons, proved to be statistically significant.

In this series of experiments there was a systematic increase in the standard deviations of the Beckman readings as the amount of antimony was increased. It will be recalled that in the determination of the standard antimony calibration

TABLE 2

The determination of antimony added to liver in the form of four therapeutically important organic antimonials compared with determinations of the same antimonials in aqueous solution

DRUG	ANTIMONY	MEAN BECKMAN READING OF CONTROL	S D *	MEAN BECKMAN READING TISSUE SAMPLE	S D *	t
	micrograms					
Stibanose	2.0	0.100	0.011	0.095	0.007	0.8
	8.0	0.495	0.014	0 498	0.022	0.2
	15.0	0.950	0.033	0.950	0.017	0.0
Neostibosan	2 0	0.076	0.008	0.084	0.008	1.6
	8.0	0 439	0.019	0.461	0.015	2.0
	15.0	0.890	0.028	0.884	0.016	0.4
Tartar emetic	2 0	0.084	0.003	0.105	0.006	7.4
	8.0	0.531	0.027	0.508	0.023	1.4
	15 0	0 992	0.054	0.956	0.035	1.2
Anthiomaline	2.0	0 091	0.009	0.090	0.006	0.2
	8.0	0.475	0.020	0.494	0.012	1.8
	15.0	0.995	0.029	0.997	0.026	0.1

$$^{*}\text{S.D.} = \sqrt{\frac{\text{Sum of squared deviations from mean}}{\text{No. of readings} - 1}}$$

curve using Sb_2O_3 the readings seemed to be equally reproducible over the range from 2.0 to 15.0 micrograms of antimony. We have no explanation for the greater reproducibility in the antimony determinations at 2.0 and 8.0 micrograms seen in the tissue antimony recovery experiments.

DISCUSSION. The quantitative estimation of small amounts of antimony was first reported by Schidrowitz and Goldsbrough in 1911 (6). Their method was based upon the extraction of antimony by boiling with copper and concentrated hydrochloric acid, the subsequent solution of the antimony and finally its conversion into the sulphide which is a highly colored salt. The estimation was made by comparing the color of the unknown with standard antimony sulphide solutions. In their published report the authors give no data but they claim that

their method gives a recovery of 70–80 per cent. The amount of antimony that Schidrowitz and Goldsbrough were interested in was about 100 micrograms.

Beam and Freak (7) were unable to confirm the accuracy of the method of Schidrowitz and Goldsbrough, but by modifying the procedure as described by the latter authors and making the colorimetric comparisons in a Dubosq colorimeter, they reported a high degree of accuracy of recovery of antimony from urine. The range of their determinations was from 300 to 1000 micrograms.

Brahmachari (8) modified Beam and Freak's method. Although his published data are too scanty to allow critical evaluation, his reported maximum error was of the order of one per cent in the range of 90–2700 micrograms.

Boyd and Roy (9), unable to confirm Brahmachari's method, adapted Gutzeit's method for arsenic determination to the estimation of antimony. This consists of the deposition of antimony sulphide on paper sensitized with mercuric chloride and comparing with standard antimony deposits. The effective range was found to be from 20 to 300 micrograms. The authors presented no experimental data in their report but stated that the error was 10 per cent.

The methods thus far mentioned have one feature in common which, irrespective of other factors such as accuracy of recovery, made them unsuitable for our purposes. *The effective range of antimony determinations in every instance is* higher than we expected to encounter in our experimental studies.

Goodwin and Page described a new technique for the determination of antimony which employs the polarograph (1). In their experimental study of the excretion of organic antimonials they had occasion to determine amounts of antimony in the range of 2 to 10 micrograms as well as larger quantities. Although they do not present data on the evaluation of the method, they state that "under favorable conditions, the error does not exceed $\pm$ 2 per cent over the concentration range 10^{-1} to 10^{-3}, $\pm$ 5 per cent over the range 10^{-3} and 10^{-4}, and $\pm$ 10 per cent over the range 10^{-4} and 10^{-5} G/100 ml." The polarographic technique has the distinct advantage over other methods in that it is possible, at least in principle, to distinguish between tervalent and quinquevalent antimony. This is particularly important in the in vivo study of the metabolism of organic antimonials. The outstanding drawback to the use of the polarographic technique for antimony determination is that one is so far limited to the examination of plasma and urine. In our experience, the presence of various as yet unidentified substances in tissue digests lead to polarographic curves which are difficult to interpret and are not necessarily reproducible.

The most recent technique described for the determination of antimony involves the detection of radioactive antimony incorporated into the desired antimony compound (10). Brady and his associates state that " in the range of 0.25 to 40 micrograms of antimony the maximum deviation was $\pm$ 3 per cent with an average deviation of 1 per cent." From this statement it would appear that this method provides greater accuracy than any other method used at the present time. The method is not practical for routine clinical use since it is necessary to synthesize the therapeutically important organic antimonials with the isotopic antimony.

Frederick (2) adapted a qualitative test for antimony with rhodamine B to a quantitative method. He presents data on both the precision and also the accuracy of Sb recovery from tissue (dried beef) of the method Unfortunately his reported evaluation of the method covered only the range of 50 to 200 micrograms of antimony. By recalculation of his results, it can be shown that from 50 to 200 micrograms of antimony the error of the method is an absolute one; the standard deviation is 2 micrograms. From his statement that below 10 micrograms of antimony the analysis results in values within 25 per cent of the true values, it would appear that a standard deviation of 2 micrograms of antimony may very well describe his analytical variation for the entire range from 1 to 200 micrograms of antimony.

Maren (3) states in his report that "on a series of digestions of blood (5 ml.), tissue, and urine with known amounts of antimony present, the extreme of variation encountered was ± 0.3 microgram" As Maren's complete data are not yet available, it is at present not possible to compare his experience and ours upon a like number and type of determinations by the rhodamine B method.

The rhodamine B method for antimony determination has several outstanding disadvantages The exact composition of the colored rhodamine B-antimony complex is not known (2) so that the kinetics and optimal conditions of the reaction can not be adequately studied. This leads to empiricism in the technique and probably accounts in part for the not inconsiderable error in precision. Also, as we have mentioned in the section on results, the calibration curve from 0 to 2 micrograms is poorly defined and the accuracy of the method in this range is undoubtedly less than in the range of 2 to 15 micrograms.

The advantages of the method are that it is simple to perform, does not require elaborate apparatus, and can be used in routine clinical examinations as well as in experimental investigations. It may be used for the determination of antimony in biological material with a precision equal to the determination of antimony in aqueous solutions Although the data presented here deal only with the recovery of antimony from liver, it has been found that the same accuracy results when the recovery is made from plasma, urine, spleen, kidney, and intestinal contents. Finally, the rhodamine B method allows the determination of antimony in a range which is more suitable for distribution studies than is possible with the majority of other reported methods.

SUMMARY

A technique for the quantitative determination of small quantities of antimony with rhodamine B has been described in detail. The precision of the method has been evaluated by making repeated determinations of antimony in the range from 2 0 to 15 0 micrograms and these results have been analyzed statistically. It has been found that within the above range the standard deviation is 0.4 micrograms of antimony. Thus, the maximum error of the technique (2 5 S.D. units) is ± 1 0 micrograms of antimony.

The recovery of four organic antimonials from liver has been compared with

their recovery from aqueous solutions and it has been found that the presence of tissue does not interfere with the determinations.

The disadvantages and advantages of the Rhodamine B method for antimony determination have been discussed.

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THE TISSUE-DISTRIBUTION AND EXCRETION OF FOUR ORGANIC ANTIMONIALS AFTER SINGLE OR REPEATED ADMINISTRATION TO NORMAL HAMSTERS[1]

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The present extensive use of organic antimonials in the therapy of various tropical diseases has led to a renewed interest in the pharmacology of antimony in this country. Although considerable information is available on the distribution and excretion of antimony (1, 2, 3), only a few studies have been made utilizing recent and reliable techniques for the determination of antimony (4, 5, 6). The material of this report presents the results of systematic studies on the distribution and excretion of 2 tervalent and 2 quinquevalent, therapeutically important organic antimonials: potassium antimony tartrate (tartar emetic), lithium antimony thiomalate (Anthiomaline[2]), diethylamino ethanol salt of sodium antimony gluconate (Stibanose[3]), and diethylamine p-aminophenylstibonate (Neostibosan[4]). The investigation was divided into three parts: 1) The distribution and excretion of antimony following a single injection, 2) The distribution and excretion of antimony following multiple injections, and 3) The distribution of antimony in chronic toxicity experiments.

I. The distribution and excretion of antimony 24 hours after a single injection.

Materials and methods. Because the results of the antimony studies were to be correlated with the investigation of chemotherapeutic agents in experimental leishmaniasis, the laboratory animal that was chosen for these studies was the hamster (*Cricetulus auratus*). Normal hamsters, about 8 weeks old, who had been maintained in the laboratory for 2 weeks or more on Rockland rat diet (D-free) supplemented with fresh greens and whole wheat bread were divided into groups of 5 at random. Following the intraperitoneal injection of the drug to be studied, the animals were placed in individual glass metabolism cages and allowed water *ad libitum*, but no food. The urine was collected under oil, and fecal contamination was prevented by a fine-meshed, copper screen which was placed close under the stainless steel, wide-meshed floor of the cage so as to minimize leaching of the feces by the urine. Since both the floor of the cage and the underlying screen consisted of metal, it was considered possible that either antimony or some other metal which would lead to erroneous determinations in the antimony technique might contaminate the urine. To test this possibility, a normal, uninjected hamster was placed in a metabolism cage for 24

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[4] Supplied by the Winthrop Chemical Company, Lot 377 AJ.

hours and the urine subsequently carried through the routine antimony determination. No antimony could be detected although the method used is sensitive to 1 microgram (7).

Twenty-four hours after the injection of the antimonial, the animals were sacrificed by severing the spinal cord in the cervical region. The organs were immediately weighed and either the entire organ or aliquots were taken for the antimony analysis. The contents of the entire intestinal tract, removed by stripping the gut, were added to the feces. This material was then dried in an oven at 100°C. for 24 to 48 hours. After the dried intestinal contents had been weighed they were ground in a mortar until a homogeneous mixture was obtained. Aliquots of the ground material were taken for antimony analysis. The urine was diluted with distilled water to a suitable volume so that the antimony analysis fell within the useful range of the method.

The amount of each drug to be injected was between 10 and 20 per cent of the murine LD50. Thus, the following schedule of doses was used: tartar emetic, 10 mgm. per kgm. body weight; Anthiomaline, 5 mgm. Sb per kgm. body weight; Stibanose, 500 mgm. per kgm. body weight; and Neostibosan, 100 mgm. per kgm. body weight. The drugs were dissolved in, or diluted (in the case of Anthiomaline) with, isotonic saline and the concentration adjusted so that 1 ml. of the drug solution was injected per 100 body weight.

The rhodamine-B method was utilized in the determination of the antimony content of the tissues, urine, and intestinal material. (All analyses were made in duplicate unless the amount of tissue available was too small.) The technique and evaluation of the method have been described in detail (7, 8). In brief, the antimony analysis on biological material consists of destruction of organic substances by digestion with concentrated sulfuric and nitric acids. Following digestion, a colored rhodamine B-antimony complex is formed in the presence of excess chloride ion. The antimony-dye complex is removed from the aqueous solution by extraction with benzol. The optical density of the benzol solution is measured in the Beckman spectrophotometer at a wave-length of 565 millimicrons, and this is compared with a previously determined calibration curve relating optical density of the rhodamine B-Sb complex to known amounts of antimony.

Results and comment. Table 1 presents a summary of the experimental findings expressed as averages, together with the standard deviation (S.D.) in each instance to indicate the range and distribution of the individual determinations about the mean. Inspection of the table reveals several distinct differences in the behavior of the 4 organic antimonials investigated. The amount of antimony injected, which was arbitrarily set at a proportion of the murine LD50, is strikingly different between the tervalent compounds tartar emetic and Anthiomaline on the one hand, and the quinquevalent antimonials, Stibanose and Neostibosan on the other. Thus more than 40 times as much antimony was injected into the animals receiving Stibanose than into those receiving tartar emetic. There is much less variation in the dosage of antimony when the comparison is made between the two tervalent compounds or the two quinquevalent compounds. This observation of the relative toxicities of tervalent and quinquevalent antimony agrees with previous clinical and experimental experience (3, 9). Because the amount of quinquevalent antimony injected exceeded so greatly the dose of tervalent antimony it was thought that the distribution of the quinquevalent antimony in the body might differ from that of the tervalent antimony merely owing to the large amount administered. For this reason another group of 5 normal hamsters was given a reduced dose of Neostibosan intraperitoneally so that the amount of antimony injected would be comparable to the dose of tartar emetic antimony. The findings in these animals are summarized in table 1 and figure 1 under Neostibosan B.

The concentration of antimony attained in the liver reveals a significant difference in the behavior of Sb^{III} and Sb^{V}. It is apparent from table 1 that the

TABLE 1

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DRUG	Sb INJ.		Sb IN LIVER		Sb IN LIVER		Sb IN SPLEEN		Sb IN SPLEEN		Sb IN KIDNEYS		URINARY Sb EXCRETION		Sb EXCRETION IN INT. CONT.	
	Avg.	S.D.	Avg. conc.	S.D.	Avg. total	S.D.	Avg. conc.	S.D.	Avg. total	S.D.	Avg. total	S.D.	Avg. total	S.D.	Avg. total	S.D.
	mgm.		*µgm./gm.*		*mgm.*		*µgm./gm.*		*mgm.*		*mgm.*		*mgm.*		*mgm.*	
Tartar emetic...	0.43	0.06	15.1	3.9	0.049	0.014	tr		tr		0.001	0.000	0.071	0.015	0.21	0.03
Anthiomaline...	0.70	0.06	27.4	2.1	0.113	0.010	tr		tr		0.002	0.001	0.110	0.025	0.37	0.05
Stibanose.......	19.10	1.04	13.5	1.2	0.050	0.003	20.2	8.0	0.002	0.001	0.015	0.002	13.9	1.3	0.61	0.42
Neostibosan A..	6.02	0.60	59.4	5.9	0.242	0.036	44.5	6.8	0.008	0.006	0.122	0.012	3.7	0.2	0.34	0.21
Neostibosan B..	0.48	0.11	2.0	1.0	0.012	0.002	tr		tr		0.017	0.005	0.292	0.036	0.031	0.016

S.D. = standard deviation.
tr = trace.

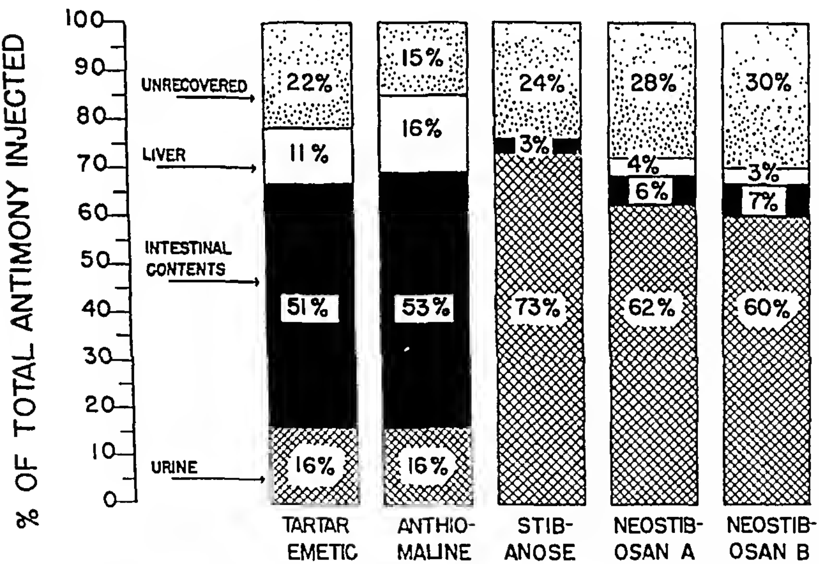

FIG. 1. THE TISSUE DISTRIBUTION AND EXCRETION OF ANTIMONY IN NORMAL HAMSTERS TWENTY-FOUR HOURS FOLLOWING A SINGLE INTRAPERITONEAL INJECTION OF FOUR ORGANIC ANTIMONIALS

The results are expressed as percentages of the total dose of antimony administered. For a discussion of the significance of the histograms labelled Neostibosan A and Neostibosan B, see the text.

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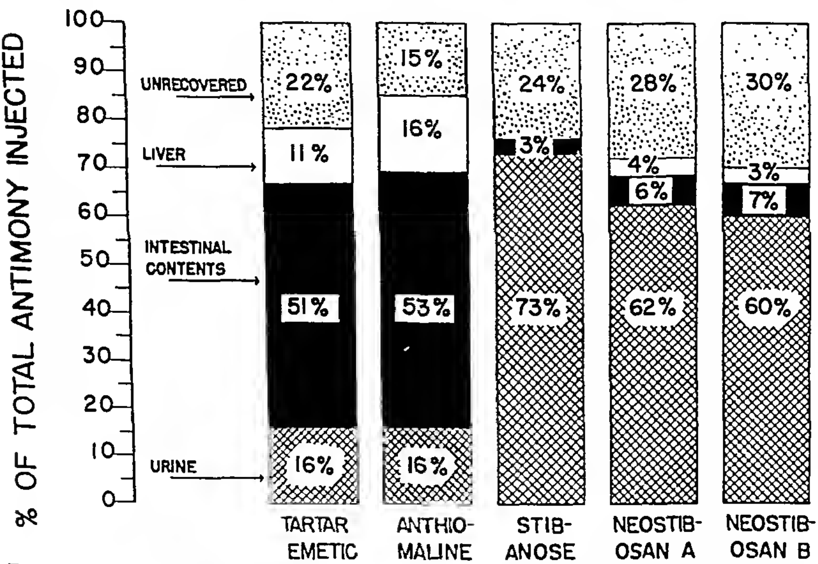

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concentration, or total amount of antimony in the liver is not primarily dependent upon the amount of antimony injected. Thus, there is no significant difference between the concentration or total mgm. of antimony in the animals receiving

tartar emetic or Stibanose, yet more than 40 times as much quinquevalent antimony was administered than tervalent antimony. Proportional to the total Sb injected, tervalent antimony localizes in the liver to a greater extent than Sb^V. It can also be seen that the reduction in the dose of Sb^V in "Neostibosan B" does not alter the discrepancy in the localization of antimony between Sb^{III} and Sb^V. Although Sb^{III} accumulates to a greater extent in the liver relative to the dosage than Sb^V, it is to be noted that valency is not the only factor determining the extent of localization of the antimony in this organ. The variation between the liver distribution of the antimony of Stibanose and Neostibosan cannot be explained on the basis of the total antimony injected or on the valency; for, although they are both quinquevalent antimonials, and even though the amount of antimony injected is smaller in the experiments with Neostibosan, there is greater antimony concentration in the liver with this drug.

The concentration and total amount of antimony in the spleen show another difference between Sb^{III} and Sb^V. Following a single therapeutic injection of the 4 drugs only traces of antimony are present in the spleen when tartar emetic or Anthiomaline are used whereas an appreciable concentration of Sb^V is attained with the other two drugs. That this is in part explicable on the basis of the total amount of antimony injected is shown by the fact that with "Neostibosan B" only traces of antimony were found in the spleen. However, as will be seen subsequently, even following repeated administration of the drugs, the difference in splenic antimony concentration between Sb^{III} and Sb^V persists. As in the case of the liver determinations, the Neostibosan antimony localizes to a greater extent in the spleen than does the antimony of Stibanose.

The total amount of antimony which is present in the kidneys following a single injection of a therapeutic dose is a small part of the total amount of antimony administered in all cases. The Sb^V of Neostibosan localizes in the kidneys to the greatest extent in absolute as well as amounts relative to the antimony dose.

The most striking differences in the behavior of Sb^{III} and Sb^V in the body are revealed in the results of the antimony excretion determinations summarized under the columns "Average Total Sb Urinary Excretion" and "Average Total Sb Excretion in Intestinal Contents" of table 1. It can be seen that the major portion of Sb^V excretion is accomplished by the kidneys, whereas the Sb^{III} is removed from the body in the intestinal contents. Although we have no unequivocal evidence as to the mechanism whereby tervalent antimony reaches the intestinal contents, the high concentration of antimony in the liver would indicate that the Sb^{III} gains access to the intestine in the bile. We have not seen any reports of studies in man in which the excretion of antimony in the intestinal contents was determined; however, Goodwin's investigation of the urinary elimination of Sb^V and Sb^{III} in man (6) and also our own determinations of the renal excretion of antimony in human patients (10) show a similar difference in the relative amount of tervalent and quinquevalent antimony excreted by the kidney. It seems likely, therefore, that in man the excretion of Sb^{III} and Sb^V in the intestinal contents is similar to that seen in our experimental animals.

In table 1 we have presented and discussed the distribution and excretion of antimony in terms of the absolute amounts of antimony involved. Figure 1 presents the distribution and excretion of antimony expressed as percentages of the total antimony injected and also shows the percentage of antimony in each instance which is unaccounted for. As has been explained above, Neostibosan A presents the results in those animals receiving an amount of drug calculated on the basis of 10–20 per cent of the murine LD50, while Neostibosan B summarizes the findings in the animals receiving a reduced dose of Neostibosan such that the total amount of Sb injected is comparable to the amount of antimony administered to the hamsters receiving tartar emetic. For practical reasons it was not feasible to represent very small percentages on the histogram so that we have omitted the per cent of the total antimony administered which is localized in the spleen and kidneys in every instance.

The difference in the method of excretion of tervalent and quinquevalent antimony can be readily seen in figure 1. It is apparent that the kidney is the principal channel for the excretion of Sb^{V}, whereas elimination of Sb^{III} in the intestinal contents is the major route of elimination. It is also to be noted that in spite of the marked difference in the dose of Sb^{V} given to the animals represented by "Neostibosan A" and "Neostibosan B", the distribution and excretion of antimony is not significantly different in the two series of experiments. Figure 1 also shows the average per cent of the total antimony administered which was not recovered. In preliminary experiments only traces of antimony were found in the brain, plasma, heart, lungs, and muscle. We therefore have discovered no major depot of antimony which would account for the substantial percentage of the metal which we failed to recover, although extensive localization in skin, bone or erythrocytes has not been ruled out.

II. The distribution and excretion of antimony after multiple injections.

Materials and methods. Because of the limitation of time and because of the large number of antimony determinations required in this experiment, it was decided to investigate only 1 tervalent antimonial, tartar emetic, and 1 quinquevalent antimonial, Stibanose. As before, the hamster (*Cricetulus auratus*) was the experimental animal Five normal hamsters of a comparable age and weight were utilized for the investigation of each drug. They were placed in individual metabolism cages and the urine and feces were collected separately. The animals were allowed food and water *ad libitum*. The drugs were injected intraperitoneally daily for 7 days, the daily dose was the same as that administered in the experiments reported in Section I, i e , tartar emetic, 10 mgm per kgm body weight; Stibanose, 500 mgm. per kgm body weight Twenty-four hours after the seventh injection, the animals were sacrificed and tissues, urine, and feces were taken for antimony analysis as has been described above

Results and comment. The antimony determinations are summarized in table 2. The results in each instance are expressed as averages together with the value of the standard deviation to describe the range of the individual determinations and also the distribution about the average.

Inspection of the findings summarized under *Liver* show a greater concentra-

TABLE 2

The tissue distribution and excretion of antimony in normal hamsters twenty-four hours after seven daily intraperitoneal injections of two organic antimony compounds

DRUG	Sb INJ		LIVER				SPLEEN				KIDNEYS		HEART				LUNGS		MUSCLE		URINE		INT. CONT	
			Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb	
	Total	S D	Conc	S D	Total	S D	Conc	S D	Total	S D	Total	S D	Conc	S D	Total	S D	Total	S D	Conc	S D	Total	S D.	Total	S D.
	mgm		μgm / gm		mgm		μgm / gm		mgm		mgm		μgm / gm		mgm.		mgm		μgm / gm		mgm		mgm.	
Tartar emetic	2 00	0 17	37 5	6 1	0 130	0 034	tr		tr		0 003	0 001	tr		tr		tr		tr		0 314	0.037		
Stibanose	120 8	6 5	77 0	11 6	0 340	0 049	60 5	13 4	0 014	0 003	0 137	0 005	2 8	1.1	0.001	0.001	0.004	0.002	2 0	0 001	113.4	12.8	1 55	0.37

TABLE 3

The tissue distribution of antimony in normal hamsters at the time of death following chronic injections of four organic antimonials

DRUG	INJ		Sb INJ		LIVER				SPLEEN				KIDNEYS		HEART				LUNGS				BRAIN				MUSCLES	
					Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb		Sb	
	No of days	S D	Total	S D	Conc	S D	Total	S D	Conc	S D	Total	S D	Total	S D	Conc	S D	Total	S D	Conc	S D	Total	S D	Conc	S D	Total	S D.	Conc	S.D.
			mgm		μgm / gm		mgm		μgm / gm		mgm		mgm		μgm / gm.		mgm		μgm / gm		mgm		μgm / gm.		mgm.		μmg / gm.	
artar emetic	22	4	11	2 4	65	24 4	0 256	0 098	14 8	10 5	0.002	0.001	0 06	0 02	5.0	1.9	0 002	0.001	8.2	1.1	0.005	0 001	2.0	1.7	0.002	0 002	4.4	1.3
nthiomaline	10	1	18	0 8	233	52 0	1.035	0.188	38 0	11.2	0 006	0.002	0.11	0 01	29.3	0.5	0 009	0.003	27.0	5.3	0 025	0 006	3.0	0.8	0 003	0 001	7.0	1.0
tibanose	27	4	752	123 0	558	35 0	1 88	0.30	1442.0	720 0	0.092	0 079	0.14	0.01	54.5	73.2	0.054	0 020	119.9	48 3	0 071	0.005	94.4	54 0	0.057	0.006	219.0	181.0
eostibosan	30	8	427	177.0	2822	1770.0	9 5	5 3	2120.0	740.0	0.138	0 042	0 33	0.18	346.0	101 0	0 127	0.030	200.0	104.0	0.120	0 035	25.0	1.3	0 020	0 002	372.0	178.0

tion of tervalent antimony relative to the total antimony administered than quinquevalent antimony. Thus, although more than 40 times as much Stibanose Sb was injected as Tartar emetic Sb, the concentration and total amount of antimony are only about two and a half times as great in the animals receiving Stibanose. However, the present findings would indicate that the *relative* rate of cumulation of antimony in the liver following repeated injections is higher in the case of Stibanose than tartar emetic.

In the 7 day experiments the total amount of Stibanose antimony in the spleen and kidneys is maintained at the same level proportional to the total amount of antimony injected that was observed following a single injection. Following multiple injections of tartar emetic only traces of antimony were found in the spleen and the total amount in the kidneys was minimal. As can be seen from table 2 only traces of antimony were detected in the heart, lungs, and muscle in the experiments with tartar emetic, and only very small amounts of antimony in the case of Stibanose. Two other tissues were also examined, plasma and brain, but with both drugs only traces of antimony could be found. As in the experiments following a single injection of an antimonial, it can be seen that the major portion of the tervalent antimony is excreted in the intestinal contents (52 per cent of the tartar emetic antimony) and 90 per cent of the Stibanose antimony is eliminated in the urine.

III. The tissue distribution of antimony in chronic toxicity experiments.

Materials and methods. As in tbe previous series of experiments, the hamster was the experimental animal. Sixteen normal animals of comparable age and weight were divided at random into 4 groups of 4 animals. Intraperitoneal injections of the 4 drugs, tartar emetic, Anthiomaline, Stibanose, and Neostibosan were given once daily until the animals died.

The initial dosage schedule was the same as that utilized in the previous experiments, i e. Tartar emetic, 10 mgm. per kgm.; Antbiomaline, 5 mgm. Sb per kgm.; Stibanose, 500 mgm. per kgm.; and Neostibosan, 100 mgm. per kgm. Witb all of the drugs it was necessary to increase the dosage because the animals showed no evidence of toxic effect as determined by weight loss. The time and change of dose will be indicated in the presentation of the results. The hamsters were weighed at weekly intervals and the dosage of the drug was adjusted according to the weight changes. The animals were allowed a normal diet and water *ad libitum.*

When death occurred, a complete autopsy was performed and tissues were secured for the determination of the antimony content. The method of analysis was that used in the other series of experiments described above.

Results and comment. Figure 2 shows the changes that occurred in the weights of the animals and also indicates the time at which the initial dosage was adjusted. As can be seen, during the first 2 weeks the daily injection of the drugs in the dosage given produced negligible changes in weight. Due to the limitation of time it was decided to increase the dosage. This change in the amount of drug injected is indicated on the graph. In the case of Anthiomaline an error was made in diluting the drug so that twelve times the initial dosage was injected rather than the planned increase of three times. This, it

is apparent, was an acutely toxic amount of the drug and all of the animals died within 48 hours after its administration. These data on Anthiomaline are included since they show that an overwhelming dose of Anthiomaline preceded by 13 smaller doses leads to no marked deposition of antimony in such organs as the heart, brain and lungs. The new dosage level for tartar emetic and Stibanose quickly proved toxic as is indicated by the progressive weight loss. It was necessary to increase the dosage of Neostibosan twice during the experiment in order to produce a continuous weight loss.

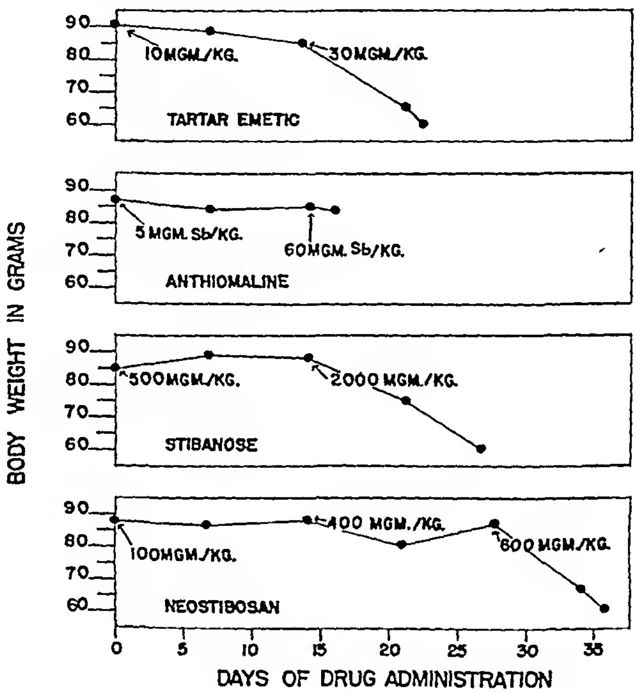

FIG. 2. THE CHANGE IN BODY WEIGHT OF NORMAL HAMSTERS RECEIVING CHRONIC INJECTIONS OF FOUR ORGANIC ANTIMONIALS PLOTTED AGAINST TIME

The final value in each instance is the body weight at the time of death. The changes in the dosage schedule of each drug is indicated.

The distribution of antimony in the various tissues of the body, together with other pertinent data, is summarized in table 3. The greater toxicity of tervalent antimonials over the Sb^{V} compounds is clearly seen when the total amount of antimony injected is compared for the four drugs. As has already been mentioned, the difference in toxicity between Sb^{III} and Sb^{V} is well known. It was hoped that an examination of the tissue localization of antimony in chronic toxicity experiments might explain the reason for this difference. We were therefore particularly interested to determine whether there was a greater concentration

of Sb^{III} in some vital structure which might account for the discrepancy in toxicity. It can be seen from table 3 that only very small amounts of antimony were found in the kidneys, heart, lungs, or brain in those animals receiving tartar emetic or Anthiomaline, and were always less than those found following the injection of the quinquevalent compounds. In these experiments as in those described in the foregoing paragraphs, the greatest localization of antimony occurred in the liver. It is to be noted, however, that the concentration or total amount of antimony present in the liver at the time of death is in no wise comparable in the experiments with the various drugs. Not only is the amount of antimony in the liver very different between the tervalent and quinquevalent drugs but even in the experiments with Stibanose and Neostibosan the amount of antimony in the liver at the time of death is markedly dissimilar.

It is interesting to note that, on the average, the per cent of the total antimony injected which was found in the liver is: tartar emetic, 2.4 per cent; Anthiomaline, 5.7 per cent; Stibanose, 0.3 per cent; and Neostibosan, 1.9 per cent. These percentages are, with the exception of Stibanose, considerably smaller than those found following a single injection.

There are two other observations in these experiments which are to be noted. It will be recalled that following the single injection of the tervalent antimonials only traces of antimony were found in the spleen as compared with the appreciable quantities which localized there in the case of the quinquevalent compounds. Similarly following a week of injections only traces of Sb were recovered from the spleen in the animals receiving tartar emetic. In the chronic toxicity experiments, too, the total amount in this organ is minimal. Attention is called to the observations because they would indicate that tervalent antimonials in non-toxic doses might not be therapeutically effective against those infections which localize to any great extent in the spleen.

The concentration of antimony in the muscle is such that, on the assumption that this tissue comprises 45 per cent of the body weight in rodents (11), 10–15 per cent of the total antimony injected would be localized in muscle.

Discussion. The experiments on the distribution of antimony in the body following single or multiple injections of tervalent or quinquevalent antimonials have clearly shown that there is a very unequal tissue distribution of the injected antimony. In the case of the tervalent compounds, tartar emetic and Anthiomaline, the liver is the only organ in which there is a considerable deposition of antimony following a single injection and even after daily injections of tartar emetic for one week the liver continues to be the only important site of antimony localization. Following the injection of the quinquevalent antimonials Stibanose and Neostibosan, the liver also retains the greatest amount of antimony, but appreciable quantities are also found in the kidneys and a high antimony concentration is present in the spleen although the total amount is low due to the small weight of the organ. The more extensive distribution of quinquevalent antimony may in large part be explicable on the basis of the very much greater amount of antimony which can be injected without causing acute toxic changes. Irrespective of the explanation for the difference in the distribution of the antimony

following administration of tervalent or quinquevalent compounds, the fact remains and these results would indicate that the quinquevalent antimonials would have greater chemotherapeutic applicability if the particular pathogenic organism is equally susceptible to either Sb^{III} or Sb^{V} compounds.

It has been found that antimony is removed from the body directly by way of the intestinal tract (most probably by biliary excretion) when tartar emetic or Anthiomaline is administered and that urinary excretion accounts for the major antimony elimination following injection of Stibanose or Neostibosan. As has been mentioned in the foregoing sections, the available evidence on antimony excretion in man indicates that the same fundamental mechanisms of antimony excretion are applicable both to the hamster and man. If this is correct, then it is clear that impairment in renal function might lead to a rapid accumulation of antimony in the body following administration of Sb^{V} compounds, and that a similar situation might exist following injection of Sb^{III} compounds in the presence of inadequate hepatic function.

The distribution of antimony in the body in the chronic toxicity experiments is disappointing in that it has not provided positive evidence to explain the greater toxicity of tervalent compounds over quinquevalent antimonials. However, the results do provide answers to a number of questions which were raised when it was decided to investigate this problem.

I. It has been established that there is no common critical concentration of antimony which must be reached in any vital organ to produce death (table 3). For example, if the value of 1 be given to the concentration in the liver at the time of death of the animals receiving tartar emetic, the antimony concentration in the liver of the hamsters receiving Anthiomaline at a comparable time is 3.6; for Stibanose, 8.6; and for Neostibosan, 44.

II. It is apparent that following chronic injections of these drugs, whether they contain Sb^{III} or Sb^{V}, there is no gradual, marked accumulation of antimony in such organs as the kidneys, heart, lungs, or brain which would unquivocally explain the lethal outcome.

III. It was considered possible that the absolute level of the antimony in vital structures might be of less importance than the rate at which the antimony accumulated in these structures. When, however, the total antimony present in the liver, for example, is plotted against time utilizing the results of the experiments after a single injection, after seven injections, and after chronic injections, it can be shown that the slope of the curves thus obtained is steepest with Stibanose intermediate with Neostibosan and lowest with tartar emetic. It must be concluded, therefore, that there is no correlation between the rate of antimony accumulation in the organs of the body and the toxicity of the compounds.

IV. Goodwin and Page (6) have observed by a polarographic technique that there was a conversion of injected Sb^{V} to Sb^{III} in the liver of rabbits and mice. By this technique it is impossible to state the proportions of the total antimony present in the liver which are in the quinquevalent and tervalent state. The method of antimony determination used in our experiments does not distinguish between Sb^{III} and Sb^{V} but does permit an accurate estimation of the total

quantity of antimony present. On the basis of our observations at the time of death following repeated injections of tervalent and quinquevalent antimonials, it would appear unlikely that the reduction of Sb^{V} to Sb^{III} takes place to a great extent or with very great rapidity because if this did occur the total amount of antimony present in the organs would be expected to be more closely alike in every instance. This is in agreement with the conclusions of Goodwin and Page.

Reasoning by analogy with the arsenicals (12) has led to the supposition that Sb^{III} compounds are more toxic because the antimony in this valency can combine directly with the sulfhydril groups of intracellular enzymes whereas the antimony of quinquevalent compounds would have to undergo preliminary reduction to Sb^{III} before toxic manifestations could appear. Although the evidence that we have obtained cannot contradict this hypothesis, the very great variation in the total amount of antimony present in the tissues at the time of death following the injection of the various drugs makes this theory less attractive. To gain an insight into the mechanism of toxicity of organic antimonials and also better to understand variation in therapeutic activity of these drugs, it would be advantageous to know to what extent the antimony dissociates from the parent molecule in the body. This knowledge is not available at the present time although Calvin and Wilson (13) have recently demonstrated that it is possible to determine this experimentally in their investigation of copper chelate compounds.

SUMMARY

Following a single injection of four therapeutically important organic antimonials, tartar emetic, Anthiomaline, Stibanose and Neostibosan, it was found that the antimony is localized to the greatest extent in the liver with all drugs; a high concentration of antimony is attained in the spleen in the case of the quinquevalent compounds, Stibanose and Neostibosan, whereas the antimony of the tervalent antimonials can only be detected in traces in this organ. This may be due to the relatively smaller amount of antimony injected in the experiments with tartar emetic and Anthiomaline which was dictated by the greater toxicity of these drugs over Stibanose and Neostibosan. The major portion (50 per cent) of the antimony of tartar emetic and Anthiomaline is excreted in the intestinal contents (probably by way of the bile), while the antimony of the quinquevalent antimonials is chiefly removed from the body by the kidneys.

Following 7 daily injections of tartar emetic and Stibanose there was an absolute increase in the total amount of antimony in the liver; however, the percentage of the total dose of antimony administered did not increase in the case of Stibanose and was smaller in the experiments with tartar emetic. In spite of the repeated doses of tartar emetic only traces of antimony were found in the spleen. The excretion of antimony in the seven day experiments was comparable to that observed following a single injection of Stibanose or tartar emetic.

In a series of experiments daily injections of the four drugs were made until chronic toxic manifestations and finally death occurred. The greater toxicity of tervalent over quinquevalent antimonials was clearly demonstrated as shown

by the larger amount of antimony that was required to produce a lethal effect in the experiments with Stibanose and Neostibosan. The tissue distribution of antimony at the time of death did not, however, afford an explanation for the difference in toxicity of the two groups of compounds. The amount of antimony in the liver (which contained more antimony than any other organ examined) varied markedly from one drug to another. Further, it was found that there was no marked accumulation of antimony in other vital organs such as the heart, brain, or lungs with any of the drugs.

The implications of the findings with respect to mechanisms of toxicity such as rate of antimony accumulation in the liver and the conversion of Sb^{V} to Sb^{III} are discussed.

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CHEMOTHERAPY OF FILARIASIS (LITOMOSOIDES CARINII) IN THE COTTON RAT BY THE ADMINISTRATION OF STIBANOSE (SOLUSTIBOSAN)

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When filaria-infected cotton rats are treated intensively with any of several antimony preparations, the adult filarias (*Litomosoides carinii*) are killed within a few days and circulating microfilariae gradually decrease in number thereafter until they finally disappear (1, 2). When certain of these drugs are administered intensively to human cases of filariasis (*Wuchereria bancrofti*), gradual and apparently permanent reduction in the microfilaria level of the blood to zero is likewise seen (3, 4), and, presumably, the adult filarial parasites in man also are killed by the drug. It appears, therefore, that the naturally-occurring filariasis of the cotton rat is a good medium for testing drugs for potential action in human filarial infection.

Since 1937, a compound of pentavalent antimony originally known as solustibosan and now called stibanose[1] has been employed to a limited extent in the treatment of leishmaniasis (5–8). It has also been tried without apparent significant effect, in a few cases of filariasis *bancrofti* (9). In the hands of all who have used it, stibanose has invariably caused only the slightest reactions, if any whatever, and it is safe to say that larger doses of stibanose, in terms of antimony, than of any other antimony compound available can be administered to patients without inducing unfavorable reactions. The drug is readily soluble in water, making a colorless solution which, unlike practically all other compounds of pentavalent antimony, can be given intramuscularly without local injury to tissues.

In order better to evaluate the potentialities of stibanose for treating human filarial infection, the authors have in a preliminary study employed stibanose to treat the filariasis of cotton rats. As will be seen from the data, it is possible to eradicate this infection from these animals by administering a few doses of stibanose.

MATERIALS AND METHODS. *The animals used.* Cotton rats naturally infected with *Litomosoides carinii* were obtained from Hegener Research Supply Company, Sarasota, Florida.

Stibanose. The stibanose was supplied either in powder form or in 6.67% solution by Dr. Justus B. Rice of the Winthrop Chemical Company, New York, through Dr. L. R. Farquhar, of the Chemotherapeutic Center, National Research Council, Washington, D. C.

[1] Stibanose is the diethylamino-ethanol-salt of sodium antimony gluconate.

Method of treatment. The infected cotton rats were injected intramuscularly during one or two weeks with different doses of drug, as indicated in the table.

Estimation of the effects of treatment. The relative number of microfilariae was estimated by counting those seen in 100 microscope fields (× 430) of fresh tail blood under a cover slip. Counts were made before treatment began and at intervals of three or four days thereafter. The treated rats were sacrificed after the intervals shown in the table, and their pleural spaces were searched for adult worms.

Results. In table 1 are shown the results of treating 12 filariated cotton rats with stibanose. Rats 1 to 5, all of which had comparatively light infections, as

TABLE 1

Effect of stibanose on filariasis of the cotton rat

COTTON RAT NO.	NUMBER OF MICROFILARIAE SEEN IN 100 FIELDS OF MICROSCOPE (× 430)							RECOVERY OF ADULT FILARIAS AT AUTOPSY†
	Day treatment began	Days after treatment began						
		1	7	14	21	35	54	
1	5	9	9	3	3		0*	10; all dead, in exudate
2	4	8	4	4	0*			15; all dead, in exudate
3	9	0	1	3	0	0*		5; all dead, in exudate
4	12	18	3	6	2	0	0*	15; all dead, in exudate
5	12	8	3	2	8	4	0*	10; all dead, in exudate
6	23	32	32	34*				100; all dead, in exudate
7	26	27	32	19	60*			60; all dead, in exudate
8	6	9	4	2	4*			10; all dead, in exudate
9	77	36	118	76	20*			35; all but 1 (male) dead in exudate
10	53	46	90	66*				100; all dead (but 2), in exudate
11	27	24	30	24*				40; 20 dead, in exudate
12	17	25	6	10*				15; 2 living, others dead in exudate

* Day of autopsy.

† When worms were matted together in exudate, numbers were approximated.

Treatment: nos. 1–7: 133 mgm. daily 6 times per week for 2 weeks; nos. 8–12: 60 mgm. daily for 6 successive days.

judged by the number of microfilariae in the tail blood, were examined at intervals following treatment until microfilariae could no longer be seen in the tail blood. All of these rats, when autopsied from 21 to 54 days after treatment was begun, harbored adult filarial worms which were dead and which were massed together in exudate. Rats 6 to 12 were autopsied 14 or 21 days from the beginning of treatment. Most of these animals had considerably more intense infections than Rats 1 to 5, and when autopsied most of them had almost as many microfilariae in the tail blood as they had before treatment began (see table 1). However, practically all the adult worms recovered from these animals were dead, and matted together in exudate (see fig. 1).

Discussion. It is apparent from the data that stibanose, like other pentava-

lent antimonials (neostam and neostibosan), exerted its action chiefly against the adult phase of the filarial worm in the cotton rat. The adult worms were killed promptly by treatment and the circulating microfilariae only slowly disappeared thereafter from the blood of rats treated with stibanose.

The chief advantage of stibanose over other antimony compounds is its comparatively low toxicity. Several cotton rats weighing approximately 200 gm. have survived single injections of as much as 0.5 gm. of drug, with no apparent difficulty, and with no reaction at the site of injection in the thigh muscle.

Because man also is known to have excellent tolerance for this drug, the trial of stibanose in human filariasis is strongly indicated. In one early trial of the drug

FIG. 1. ADULT FILARIAL WORMS (*Litomosoides carinii*) RECOVERED FROM PLEURAL SPACE OF COTTON RATS TREATED WITH STIBANOSE
Note that most worms are massed together in fibrinous exudate.

by previous investigators (9), microfilariae were temporarily diminished in number during the very brief period that four patients were followed after comparatively light treatment. It seems possible that better results would be obtained through intensive treatment provided that patients were observed for long periods thereafter.

CONCLUSION

The filaria (*Litomosoides carinii*) of the cotton rat can be eradicated from these animals by the repeated administration of the pentavalent antimony compound stibanose (or solustibosan). Because of the excellent tolerance man is known to have for this drug, its trial in the chemotherapy of human filariasis is strongly indicated.

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THE SITE OF ACTION OF NARCOTICS ON BRAIN METABOLISM[1]

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According to the theory of narcosis proposed by Quastel and associates (1, 2) narcotics exert their effect by inhibiting certain metabolic processes in brain required for the metabolism of carbohydrate, upon which brain is largely dependent.

That this is a possible explanation of narcosis is supported by the findings of various investigators showing that there is a parallelism between *in vitro* and *in vivo* effects. Quastel and Wheatley (3) found that among narcotics of the same chemical type those with the greater hypnotic activity had the greater inhibitive power on *in vitro* respiration. Fuhrman and Field (4) investigated the relationship between chemical structure and inhibiting action of barbituric acid derivatives on rat brain respiration and noted certain parallelisms between physical properties considered to be related to the production of narcosis, and biological action.

The site of action of narcotics has not been determined. Keilin (5, 6, 7) in his classical work on the cytochromes found that narcotics (urethane in particular) prevented the oxidation of cytochrome b by the other components of the cytochrome system. He used rather high concentrations of narcotic (0.1–0.5% urethane), and investigated the effect of narcotics on the oxidation of succinate. On the basis of his experiments he suggested that the effect of narcotics consisted in bringing about the formation of a not easily dissociable complex composed of dehydrogenase, substrate and cytochrome b. Jowett and Quastel (8, 9) investigated the effect of ether, chloretone and evipal on brain metabolism and found that certain concentrations which inhibited markedly the oxidation of glucose, lactate and pyruvate had no effect on the oxidation of succinate or of p-phenylene diamine. Since, in the oxidation of succinate no coenzymes are required, and hydrogen is transferred directly by means of succinic dehydrogenase to the cytochrome system, the absence of effect of narcotics on the oxidation of this substrate indicated that the cytochrome system, or that part of it involved in the oxidation of succinate, was relatively resistant to the action of narcotics. Davies and Quastel (10) showed that the anaerobic oxidation of glucose, fructose and mannose by brain tissue could be inhibited by narcotics, but that the concentration required was considerably higher than that required for inhibition under aerobic conditions. These results showed that the dehydrogenase was relatively insensitive to the action of narcotics. Michaelis and Quastel (11) narrowed the narcotic range to a region represented in the following scheme (see Ball 12).

[1] This work was supported by a grant from the Mallinkrodt Chemical Works.

SCHEME I

Narcotic insensitive region:

$\frac{1}{2}O_2 + H_2 \longrightarrow H_2O$
↑
cytochrome oxidase (1 e)
↑
cytochrome a (1 e)
↑
cytochrome c (1 e)

Narcotic sensitive region:

↑
cytochrome b (1 e)
....↑.................................... II
flavoprotein 2 e* + $2H^+$
....↑.........H_2.......................... I
cozymase (pyridine nucleotides)

Narcotic insensitive region:

↑ H_2
dehydrogenase
↑
substrate

* e = electron.

Accepting Quastel's results there are two possible positions where narcotics may exert their inhibition—(I) by blocking the transfer of H from cozymase to flavoprotein and (II) by blocking the transfer of electrons from flavoprotein to cytochrome b. If the inhibition occurs at I in the above scheme one would expect to find an accumulation of reduced cozymase (coenzyme I) in the presence of narcotic. One would also expect to find that the transfer of hydrogen from reduced cozymase by flavoprotein to some suitable hydrogen acceptor was blocked by the addition of narcotics.

If the above scheme represents a complete picture of the metabolic pathway in brain and if the block does *not* occur at I, by the process of elimination it must occur at II. There still remains the possibility, however, of another step in place of, or in addition to cytochrome b, between flavoprotein and the rest of cytochrome system (see e.g. 13, 14, 15). Further information on the position of the block may be obtained by determining the effect of narcotics on an enzyme requiring the cytochrome system but not cozymase. Such a system is that found in yeast, which oxidized lactic acid. In contrast to the animal lactic enzyme which requires cozymase the yeast lactic enzyme does not require a coenzyme but transfers hydrogen by means of the dehydrogenase and the cytochrome system. If such a system is inhibited by narcotics it would be inferential evidence that the narcotic block was not at I, and that it could occur at II. The line of attack outlined above was pursued, and the results are presented below.

EXPERIMENTAL. *Methods.* Measurement of oxygen consumption was carried out in Warburg manometers at 37°C.

Ringer-phosphate buffer of the following composition was used unless otherwise stated: NaCl 0.13 M, KCl 0.002 M, $CaCl_2$ 0.001 M, sodium phosphate buffer pH 7.4, 0.03 M. The final concentration of substrate was 0.026 M. The concentration of nembutal unless otherwise stated was 100 γ/cc.

In the experiments with brain, whole rat brain was homogenized in buffer in a glass homogenizer obtained from the Scientific Glass Company, and was diluted with buffer to

give a concentration of 300 mgm. wet tissue per cc. One half cc. of the suspension, thus prepared, was measured by means of a tuberculin syringe into a Warburg vessel of approximately 15 cc. capacity. The total volume of fluid in each vessel, exclusive of KOH in the centre well, was 1.5 cc.

In experiments with yeast Fleischmann's baker's yeast was used. It was washed once with a large quantity of distilled water (about 200 cc. per gm. yeast), centrifuged and suspended in the desired medium.

Cozymase was prepared from Fleischmann's baker's yeast by the method of Williamson and Green (16) and reduced cozymase by the method of Green and Dewan (17). Cozymase was determined using the method of Axelrod and Elvehjem (18) by means of apozymase prepared from brewer's bottom yeast as already described (19). Reduced cozymase was determined by the method of Adler, Hellström and v. Euler (20). The presence of nembutal did not affect these determinations. Flavoprotein was prepared according to Lockhart (21). In Thunberg experiments the methylene blue solution (0.07%) was placed in the hollow stopper, the other components in the tube (see e.g. 22). The tube was evacuated and flushed out with N_2. This process was repeated and the tube finally evacuated and the stopper closed. The tube was warmed for two minutes in a bath at 37° before mixing.

Results. Nembutal and chloroform were found not to inhibit the oxidation of succinate. Therefore the cytochrome system, or that part of it involved in the oxidation of succinate was not affected by these narcotics. The oxidation of glucose, lactate and pyruvate was, however, inhibited (table 1).

That the dehydrogenases were not affected by nembutal was shown by the finding that methylene blue could by-pass the block produced by narcotic (table 1). Leuco methylene blue, being autoxidisable in air, can serve as a H carrier between the dehydrogenase and oxygen, as illustrated below.

$$\text{Leuco methylene blue} + O_2 \rightarrow \text{Methylene blue} + H_2O$$

$$\text{Substrate} + \text{methylene blue} \xrightarrow{\text{dehydrogenase}} \text{oxidised substrate} + \text{leuco methylene blue}$$

Nembutal and chloroform thus resemble other narcotics investigated, in their effect on brain metabolism.

In order to determine whether the nembutal block is at position I (scheme I) the experiments described below were carried out.

(a) The first experiment was designed to determine whether the presence of nembutal caused the accumulation of reduced cozymase during the metabolism of carbohydrate by brain. Brain tissue was allowed to metabolize lactic acid in the presence and in the absence of nembutal for one hour, at the end of which time reduced cozymase was determined. The results given in table 2, show that nembutal does not cause an appreciable accumulation of reduced cozymase.

(b) To determine whether nembutal inhibited the reaction,

$$\text{reduced cozymase} + \text{methylene blue} \xrightarrow{\text{flavoprotein}} \text{cozymase} + \text{leuco methylene blue}$$

experiments were carried out in Thunberg tubes as described in the section on methods. The rate of the reaction was determined by measuring the time required for the decolorization of methylene blue by reduced cozymase in the

presence of flavoprotein under anaerobic conditions. The results of these experiments (table 3) show that the above reaction is not affected by nembutal.

These two groups of experiments indicate that the block produced by nembutal in the metabolism of carbohydrate does not occur at position I (scheme I).

TABLE 1

The inhibition of carbohydrate metabolism by narcotics and the reversal of the inhibition by methylene blue

Each Warburg vessel contained 150 mgm. homogenized brain with the additions indicated, in a total volume of 1 5 cc.

SUBSTRATE 0 026 M	NARCOTIC*	METHYLENE BLUE 6 × 10⁻⁵ M	DURATION OF EXPERIMENT	MM $^3O_2$		INHIBITION
				Without narcotic	With narcotic	
			min			%
glucose	nembutal	−	40	87	61	30
lactate	nembutal	−	40	99	60	39
succinate	nembutal	−	40	170	180	0
glucose	chloroform	−	70	243	167	31
succinate	chloroform	−	70	354	350	0
glucose	nembutal	−	60	173	72	58
pyruvate	nembutal	−	60	332	109	67
glucose	nembutal	−	70	171	83	51
pyruvate	nembutal	−	70	319	113	65
succinate	nembutal	−	60	296	274	7
pyruvate	nembutal	−	105	337	221	35
pyruvate	nembutal	+	105	391	385	0
pyruvate	nembutal	−	75	172	78	55
pyruvate	nembutal	+	75	277	251	9
glucose	chloretone	−	75	202	139	31
glucose	chloretone	+	75	326	330	0
pyruvate	chloretone	−	85	321	206	36
pyruvate	chloretone	+	85	370	375	0
glucose	chloroform	−	60	222	144	35
glucose	chloroform	+	60	304	295	0

* The concentrations of narcotic used were as follows Nembutal 100 γ per cc , chloretone 330 γ per cc , and $CHCl_3$ 0 6 cc. buffer solution saturated with chloroform in each vessel.

The effect of nembutal on the oxidation of lactate by yeast was next investigated. The yeast enzyme oxidizing lactate, as stated above, does not require cozymase for its action. The experimental results given in table 4 show that nembutal inhibits the oxidation of lactate by yeast. The effect is the same in

the presence or in the absence of semicarbazide which would bind the pyruvate formed from the oxidation of lactate. The inhibition produced by nembutal is not due to any effect on the dehydrogenase since the anaerobic reduction of methylene blue by yeast lactic dehydrogenase was not inhibited but was, in fact, somewhat accelerated by nembutal (table 5). Since cozymase is not involved in the oxidation of lactate by yeast, and since the action of the dehydro-

TABLE 2

Reduced cozymase formed by normal brain and brain inhibited by nembutal

Brain homogenate (150 mgm.), lactate (.026 M), cozymase (about 450 γ), and nicotinamide (3 mgm.), were shaken with and without nembutal at 37° for one hour in Warburg vessels. The contents were then heated at 90° for five minutes to destroy the enzymes, and centrifuged. The pH of the supernatant fluid was then adjusted to 10 and the mixture heated at 100° for fifteen minutes. This destroys cozymase but has no affect on reduced cozymase. The pH was adjusted to 6 for the analysis.

O_2 UPTAKE (mm^3)		REDUCED COZYMASE (γ)	
Without nembutal	With nembutal	Without nembutal	With nembutal
306	95	9.8	11.7
225	130	6.9	5.6
368	111	6.3	8.5

TABLE 3

Effect of nembutal on the oxidation of dihydrocozymase

Contents of Thunberg tubes		
Without reduced cozymase	*With reduced cozymase*	*With reduced cozymase and nembutal*
0.5 cc. blank*	0.5 cc. dihydrocozymase	0.5 cc. dihydrocozymase
0.5 cc. methylene blue	0.5 cc. methylene blue	0.5 cc. methylene blue
1 cc. flavoprotein	1 cc. flavoprotein	1 cc. flavoprotein
1.5 cc. buffer	1.5 cc buffer	1.1 cc. buffer
		0.4 cc nembutal
Time *for decolorization of Methylene blue*		
Average of three experiments		
min	*min*	*min*
60	10.6	11.6
Average of three experiments using dialysed flavoprotein		
150	70	70
Average of two experiments		
210	44	39

* The blank contained the reagents used in the reduction of cozymase.

genase is not inhibited by nembutal, the block must by elimination, occur at position II or at some point with a similar redox potential.

Discussion. It has been shown that the narcotics ether, chloretone, luminal (1, 2, 3, 8, 9, 10, 11), nembutal and $CHCl_3$ (present paper) inhibit the oxidation of glucose, lactate and pyruvate but have no effect on the oxidation of succinate. This effect of narcotics would seem to be quite a general one. These results show that that part of the cytochrome system required for the oxidation of succinate

is relatively insensitive to the action of narcotics. It has also been shown that the dehydrogenases are unaffected by narcotics except in high concentrations. If the dehydrogenases and the cytochrome system are insensitive to narcotics, at least two positions remain which could be affected. These are indicated in scheme I. These two possibilities have been investigated by several different methods, and we have found:

TABLE 4

The effect of nembutal on the oxidation of lactate by yeast

0.5 cc. yeast (1 g. in 30 cc.) was used in each vessel

MEDIUM	DURATION OF EXPT	SUBSTRATE	NEMBUTAL	MM $^3O_2$ CONSUMED		INHIBITION
				Without narcotic	With narcotic	
	min		γ/cc			%
Water	120	lactate	400	196	143	26
NaCl	120	lactate	400	184	82	56
Water	115	———	400	38	61	0
Water	115	lactate	200	217	169	22
Water	115	lactate	400	217	100	54
Ringer-phosphate buffer	45	lactate	400	120	80	34

TABLE 5

Effect of nembutal on the lactic dehydrogenase of yeast

	Contents of Thunberg tubes: Without nembutal	Contents of Thunberg tubes: With nembutal
	0.5 cc. methylene blue (0 0703%)	0.5 cc. methylene blue
	1 cc. yeast suspension	1 cc. yeast suspension
	0.2 cc lactate M/5	0 2 cc. lactate
	1 3 cc water	0 4 cc. nembutal (600 γ)
		0.9 cc. water
	Time for reduction of methylene blue in minutes	
Experiment 1*	34, 35	15, 15
Experiment 2†	25, 27	7, 7

* Yeast suspension contained 1 gm. in 100 cc. water.

† Yeast suspension contained 3 gm. in 100 cc. water.

(1) That there was no accumulation of reduced cozymase in the presence of nembutal when brain tissue metabolized lactate.

(2) That nembutal did not interfere with the reaction

$$\text{reduced cozymase} + \text{methylene blue} \xrightarrow{\text{flavoprotein}} \text{cozymase} + \text{leuco methylene blue.}$$

These results indicate that nembutal does not exert its effect by blocking metabolism at position I. Further substantiation of this is found in the fact that the

lactic enzyme in yeast, which does not require cozymase, was inhibited by nembutal. Quastel and Wheatley found that the residual respiration of yeast was unaffected by narcotics which is in agreement with our results. They, however, did not determine its effect with added substrate. If the above scheme is a complete representation of the metabolic pathway, the only position remaining where narcotics can exert their inhibition is at cytochrome b. Keilin (5, 6, 7) using high concentrations of urethane which inhibited the oxidation of succinate suggested that the narcotic bound the dehydrogenase, substrate and cytochrome b into an inactive complex. By means of a spectroscope he was able to see that the absorption band produced by reduced cytochrome b persisted on aeration in the presence of narcotic. Evidence that the narcotic bound the dehydrogenase, substrate and cytochrome b was provided by the observation that when reduction of cytochrome b was effected in the presence of urethane by sodium hydrosulphite instead of dehydrogenase + substrate the band of reduced cytochrome b did not persist after the disappearance of the other reduced cytochrome bands. Similar effects were observed by Tamiya and Ogura (23). It is not known whether lower concentrations of narcotic which will inhibit lactate and glucose oxidation will produce visible effects in the rate of disappearance of the band of reduced cytochrome b as compared with bands of the other cytochromes.

It has not been definitely proven that cytochrome b is the intermediate between flavoprotein and cytochrome c, and between yeast lactic dehydrogenase and cytochrome c, but there is evidence that some intermediate does exist (13, 14, 15), and it must have a redox potential similar to that of cytochrome b. Regardless of whether it is cytochrome b or some other as yet unidentified intermediate, which transfers hydrogen at this particular step, it seems to be fairly definite that it is at this stage that narcotics exert their effect in inhibiting carbohydrate metabolism of brain. It is possible that the concentration required to produce inhibition is a matter of the affinity of the particular narcotic in question for binding the flavoprotein-cytochrome b (or other intermediate) complex, or the succinic dehydrogenase-cytochrome b complex. The affinity of the narcotic for the enzyme in the former case might be greater than in the latter and a lower concentration of drug would then be effective.

SUMMARY

Previous experiments have shown that the dehydrogenases and that part of the cytochrome system involved in the oxidation of succinate are relatively insensitive to narcotics. Two possible oxidation-reduction reactions on which narcotics might exert their effect in inhibiting the carbohydrate metabolism of brain, remain. They are

(1) The transfer of hydrogen from reduced cozymase to flavoprotein and

(2) the oxidation of flavoprotein by the cytochrome system by means of electron transfer.

These possibilities have been investigated here and it has been found that re-

duced cozymase did not accumulate during the carbohydrate metabolism of brain in the presence of nembutal; and that the reaction

$$\text{reduced cozymase} + \text{methylene blue} \xrightarrow{\text{flavoprotein}} \text{cozymase} + \text{leuco methylene blue}$$

was not affected by nembutal.

These results indicate that the block was not at the position suggested as the first possibility.

The oxidation of lactate by yeast was also found to be inhibited by nembutal. The fact that the yeast lactic enzyme, which unlike the enzyme in animal tissues does not require cozymase for activity, was inhibited, is further evidence that cozymase is not involved, and that the block occurs at cytochrome b or at some as yet unidentified step having properties similar to cytochrome b. We suggest that the narcotic may act by binding the reduced flavoprotein with cytochrome b (or other intermediate) and that the affinity of narcotic for this complex is greater than for the succinic dehydrogenase-cytochrome b complex which is not affected by low concentrations of narcotic.

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THE ACTION OF SODIUM SALICYLATE AND SULFADIAZINE ON HYALURONIDASE

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In his review on tissue permeability and the spreading factors in infection, Duran-Reynals (1) has presented the literature on connective tissue permeability and its rôle in the spread of bacterial infection and the resulting inflammatory reaction. It has been shown that in various forms of connective tissue there is a viscid ground substance which is the final product of a secretion of the connective tissue cells. From the ground substance there has been isolated a polysacchide, hyaluronic acid. The ground substance acts as a barrier to penetration and spread of foreign matter, but under certain circumstances this blocking action is absent and the foreign substances applied are distributed over a wider area.

In bacterial and tissue cells, particularly testicular cells, there is found an enzyme, hyaluronidase, which hydrolyzes and degradates hyaluronic acid, decreasing its viscosity and allowing an increase in permeability and spread of foreign material through connective tissues.

It is known that in rheumatic fever the mesenchyme tissues are especially involved and the rapid spread through these of the infecting agent and its inflammatory sequence evidence a removal of the protective connective tissue ground substance barrier. The recognized effectiveness of salicylic acid in arresting the inflammatory process and causing regression of symptoms in this disease has not been explained. As it is not considered a bacteriostatic agent, evidence of any relationship to one or other of the spreading factors should be of great interest.

In the work here reported the influence of sodium salicylate on one of the spreading factors, hyaluronidase, has been studied, and a comparison made with that of a bacteriostatic agent, sulfadiazine.

METHOD. In techniques for studying the effects of hyaluronidase as a spreading factor the general principle is to inject intradermally colored substances, such as haemoglobin, trypan blue, India ink (2), whose range of distribution can be readily seen, or those whose effects can be observed visually, for example, the spread of inflammatory reaction from diluted diphtheria toxin (3), the time for disappearance of the wheal from saline injection (4).

In the procedure adopted by us, Higgins India ink was injected into the skin of rabbits. We have selected this dye as an indicator of the spreading phenomenon because in our work it has given consistently uniform values. Four skin areas in each rabbit were taken. In one area a solution of the ink in physiological saline was injected intradermally and the rate and extent of its spread measured. Into a second area the intradermal injection was then repeated with hyaluronidase added to the India ink solution, and its effects in increasing the spread measured. Into the third and fourth areas the India ink solution alone or with hyaluronidase was injected after preliminary administration of either sodium salicyl-

ate or sulfadiazine.[1] The salicylate in 0.07 gm. or 0.10 gm./kgm. in 10% solution was injected intravenously fifteen minutes before the intradermal dye solution. The sulfadiazine, 1% in gum arabic emulsion, 10 cc./kgm., was given by stomach tube four hours prior to the intradermal tests. Throughout all the experiments the measurements of skin areas through which the ink permeated were made at one and twenty-four hours after the test injection.

The skin areas receiving the ink were measured by tracing the spread on cellophane and by transparency on millimeter ruled paper, calculating the area in mm². The error using this method was within ± 5 mm². All figures including standard deviation, fiducial limits and standard error are recorded in actual values of mm.² and not in logarithms.

The determination of parameter values which characterize this population is based on:

a. Actual value in mm.² in each animal........................ x
b. Sum of the values in mm.² in each group........................ Sx
c. Number of cases.. n
d. Mean of group in mm.².. $\bar{x} = \frac{Sx}{n}$
e. Sum of the squares of the deviation from the mean.............. $[x^2] = S(x^2) - \bar{x}\,(Sx)$
f. Variance.. $V = \frac{[x^2]}{n-1}$
g. Standard deviation.. s
h. Standard error of the mean.. $s_{\bar{x}} = \sqrt{\frac{S^2}{n}}$
i. Fiducial limits with probability of 0.5 and $n - 1 = 5$: F.L. $= \bar{x} \pm s_{\bar{x}}(t)$ according to the t value 2.571.

The hyaluronidase used was obtained by extraction from powdered bull testicular extract. The extract was made in June of 1942 and its activity determined by titration periodically to ascertain its actual strength. At the time of use it had the same activity as at the time of preparation. To decrease the amount per injection, it was used as a 2% solution of the hyaluronidase in 0.9% physiological saline, instead of the 1% solution employed by Cahen and Granier (5). Excessive shaking was avoided to prevent the inhibition of the enzyme. The testicular extract was centrifuged at 2000 revolutions per minute for 5 minutes and the clear supernatant liquid was drawn off as solution for injection.

A total of 25 New Zealand albino rabbits were used for the experiment. They were from the same stock, having weights varying between 1.5 and 2.0 kilograms. They were divided into four groups of six each: one animal was used for trial purposes to establish dosage and time of reading. In order to avoid the weight factor errors which might influence the results, and because the ventral skin and that of the groin and axillar regions afford better diffusibility, and also because of the antigenic nature of the preparation employed, the following procedure was adopted.

Four basic experiments were conducted on each rabbit. The dorsal skin was divided into four areas; two anterior, right and left behind the folds of the anterior extremities on both sides of the vertebral column, and two posterior, one right and one left in front of the folds of the posterior extremities.

In accordance with this plan, four groups of six animals each, two groups for the study of salicylate action and two groups for the study of the action of sulfadiazine, received the inoculation in the following order of Latin squares

A B D C
and
C D B A

A represents the intradermal inoculation of 0.25 cc. of 0.9% sodium chloride solution + 0.25 cc. of India ink diluted 1:2 in physiological saline.

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[1] Sodium salicylate from Mallinckrodt Chemical Works and sulfadiazine from Calco Chemical Division were employed.

B represents the administration of the drug, sodium salicylate or sulfadiazine, followed by 0.25 cc. of 0.9% sodium chloride solution + 0.25 cc. of a 1:2 dilution of India ink, in intradermal injection.

C represents the intradermal inoculation of 0.25 cc. of 2% hyaluronidase in physiological saline + 0.25 cc. of a 1:2 dilution of India ink in physiological saline.

D represents the previous administration of the drug followed by the intradermal inoculation of 0.25 cc. of 2% hyaluronidase in saline + 0.25 cc. of 1:2 India ink in saline suspension.

Results. Under the influence of the hyaluronidase preparation in control areas (C), twenty-four determinations gave diffusion areas of 3977 ± 171 mm.² This value is six times greater than that area obtained in the same animal (A) which did not receive hyaluronidase. The areas of this group measured 676 ± 30 mm.²

TABLE 1

Latin squares

	A B C D				A B C D				D C B A				D C B A			
	Group 1, Sodium salicylate				Group 2, sulfadiazine				Group 3, Sodium salicylate				Group 4, sulfadiazine			
	A	B	C	D	A	B	C	D	B	A	D	C	B	A	D	C
Parameters at 1 hr.																
Total area—Sx_1	3860	3015	16200	8460	3970	4015	16345	14725	2835	4030	6725	20285	3725	3715	20715	18535
Mean area—$\bar{x}_1$	643	502	2700	1413	662	669	2724	2454	472	672	1121	3381	622	619	3452	3089
Standard deviation—s_1	±63	53	230	198	26	64	334	174	26	62	110	177	29	35	302	220
Standard error—$s_{\bar{x}_1}$	±26	22	94	81	11	26	136	71	11	34	45	72	12	14	123	90
Parameters at 24 hrs.																
Total area—Sx_2	4120	3385	29100	10990	4175	4450	31100	30985	3050	4455	10090	29580	4045	4205	27625	28120
Mean area—$\bar{x}_2$	687	564	4850	1832	696	742	5183	6164	609	742	1681	4930	674	701	4604	4687
Standard deviation—s_2	±67	65	251	101	25	27	215	270	44	63	123	243	53	40	437	341
Standard error—$s_{\bar{x}_2}$	±28	22	102	41	14	11	88	110	18	26	50	99	22	16	179	139
Combined mean—$\bar{x}$	665	533	3775	1622	674	705	3953	3809	490	707	1401	4155	648	660	4028	3888
Fiducial limits—F. L. (19 in 20)	±69	57	226	167	31	46	288	234	39	77	123	221	44	39	386	296
Standard deviation—s	±65	54	240	150	26	46	225	222	35	73	116	210	41	25	226	167
Percentage	100	80	100	43	100	105	100	96	69	100	34	100	98	100	103	100
Variability	±9.8	10.2	6.4	9.2	3.8	6.5	6.7	6.1	7.1	10.3	8.3	5.1	6.3	3.8	5.6	4.2

The results of all experiments conducted are listed in table 1. Although the control areas (B) that did not receive hyaluronidase appear to have been reduced 20% with the administration of sodium salicylate in dosage of 0.07 gm./kgm. and 31% in dosage of 0.10 gm./kgm., the D areas which received hyaluronidase show a reduction of 57% with the administration of 0.07 gm./kgm. salicylate and almost 66% with 0.10 gm./kgm. The effect of the salicylate appears promptly in each case.

The variations observed in the spreading area either in groups two and four which may (D) or may not have received hyaluronidase (B) after prior treatment with sulfadiazine are not significant since all differences fall into the coefficient of variability. It is important to point out that with the administration of

sulfadiazine, the reading of the skin areas after 24 hours is made difficult in many instances by an equivalent inflammatory zone. According to Duran-Reynals, (1) this phenomenon is to be interpreted as favoring spread in connective tissue.

DISCUSSION. The inhibition of hyaluronidase by sodium salicylate in so typical and intense a manner and the ineffectivity of sulfadiazine in this respect may help to explain the mechanism of the sodium salicylate in rheumatic fever.

Rheumatic fever is a disease of the mesenchyma characterized not by the virulence of the etiologic organism but by its invasiveness, expecially in the young. As shown by Bensley (6) the connective tissue in formation passes progressively to the adult stage through the following phases: oedema, gelatinous ground substance, argyrophilic fibers, and collagen. In the early stages, diffusibility in the ground substance is at its maximum, while the collagenous fibers that characterize adult connective tissue retard diffusibility since the ground substance has been replaced by the fibers. The importance of hyaluronidase in the spreading factor of connective tissue is based on the observation of Meyer and Palmer (7) who pointed out that the principal substrate of connective tissue and mucoid structures is hyaluronic acid composed of equimolar parts of d-glucosamine, glycuronic acid, and N-acetyl glucosamine. The ground substance in the regions affected by rheumatic fever, such as articulations and synovial fluid, is composed practically entirely of hyaluronic acid.

Duran-Reynals (8), Kendall and associates (9) have observed that several microorganisms including the streptococci produce, or possess, hyaluronidase in their capsules. The hyaluronidase of bacterial origin or of testicular extract as prepared and used in this work increase the spread by means of enzymatic activity hydrolizing the hyaluronic acid present in the ground substance, decreasing its viscosity, and thus favoring the passage of liquids and pathogenic microorganism. The action of hyaluronidase may be divided into the following stages,

a. decreased acetic acid coagulation of the substrate
b. decreased viscosity
c. hydrolysis of hyaluronic acid with the release of glucosamine and reducing substances.

The sodium salicylate acts as an inhibitor of the enzymatic action of hyaluronidose and thus tends to limit spread in connective tissue.

SUMMARY AND CONCLUSIONS

In the presence of a 2% purified testicular extract containing hyaluronidase, the spread areas of India ink diluted 1:2 with saline were six times greater than in control animals, rabbits being employed.

The administration of sodium salicylate inhibited the spreading effect of hyaluronidase. The degree of inhibition varied with the dose of salicylate administered.

Sulfadiazine did not reduce the activity of hyaluronidase, but appeared to enhance its effect in several groups.

The significance of the inhibition of hyaluronidase by sodium salicylate in rheumatic fever is discussed.

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DIGITALIS

II. A Chemical Evaluation of Tablets of Digitoxin*

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In view of the proposed admission of digitoxin and tablets of digitoxin to the forthcoming revision of the United States Pharmacopoeia this study was undertaken to develop a method for the assay of the tablets.

Our attention has been directed especially to the Baljet color reaction (1) which appeared to have considerable promise. In 1922 Knudsen and Dressbach (2) reported a colorimetric method of assay of digitalis preparations based upon this color reaction in which a red color is developed by the active digitalis glycosides in the presence of an excess of alkaline sodium picrate. Some years later Lendle and Schmelser (3) recommended the reaction, which they attributed to the presence of unsaturated lactone groups, as a basis for a reliable method for the determination of digitalis substances. They recognized that the reaction is not specific and that sugars could interfere. Recently, the present authors (4) presented a colorimetric method for the assay of digitalis preparations which was based on the Baljet reaction and which was found to be satisfactory.

In applying the method to the assay of digitoxin tablets two main steps are involved: (1) efficient extraction of the digitoxin from the tablet by a suitable solvent and (2) treatment of this extract for the development of the color and subsequent colorimetric analysis. With regard to the first step we have restricted our consideration to the two dosage forms of the tablets which are now commercially available, viz., 0.1 mg. and 0.2 mg., and we have arbitrarily selected as the basis of the assay the number of tablets containing a total of 2 mg. of digitoxin, i.e., 20 tablets of the 0.1 mg. dosage and 10 tablets of the higher dosage form.

For the extraction of the digitoxin from the tablets, three of the more common solvents are available: chloroform, ethyl alcohol, and methyl alcohol. The use of chloroform was avoided since a solvent miscible with water is required in the final step of the color reaction and the use of this solvent would therefore involve additional steps of evaporation and filtration. The solvent selected was absolute methyl alcohol since it appears to dissolve digitoxin more readily than absolute ethyl alcohol and since it is more readily available in reagent grade. Preliminary experiments showed that the digitoxin is efficiently extracted by this solvent from the powdered tablets by mechanical shaking at room temperature, and a clear extract is readily obtained from the mixture by centrifuging.

The procedure for developing the typical Baljet color reaction in the methyl alcohol extract of the tablets presented only one serious difficulty. The final

* The expense of this investigation was defrayed in part by a grant from the Board of Trustees of the United States Pharmacopoeial Convention.

mixture must contain sufficient water to avoid the precipitation of sodium picrate and at the same time, contain sufficient alcohol to avoid precipitation of the digitoxin. Fortunately, we were able to find a methyl alcohol-water ratio which satisfied both of these requirements. We have also determined the time interval required for full development of the color reaction. This interval is appreciably longer than that required for digitalis preparations, a fact that can be readily explained on the basis that the color reaction develops more rapidly in aqueous solutions than in those of high alcohol content. The transmission of the solutions was determined with the aid of the Electrophotometer of the Fisher Scientific Co.

The final procedure which we have established is as follows: Grind up to a rather fine powder in a mortar the number of digitoxin tablets expected to have a total digitoxin content of 2 mg. Transfer the powder to a 125 cc. glass stoppered Erlenmeyer flask, add from a burette exactly 40 cc. of absolute methyl alcohol shaking during the addition to avoid the formation of lumps. Stopper the flask and shake mechanically for 15 minutes. Transfer the mixture to a centrifuge tube, cover the tube and centrifuge until a clear separation is obtained. Pipette off very carefully 25 cc. of the clear supernatant liquid. Transfer to a suitable container and, noting the time, add exactly 25 cc. of picric acid reagent freshly prepared by diluting 50 cc. of absolute methyl alcohol containing 1 gram of picric acid with 5 cc. of 10% sodium hydroxide and then water to a final volume of 100 cc. After 30 minutes measure the transmission of the solution with a photoelectric colorimeter using an absorption cell having a light path of 50 mm. and a green filter with peak transmission in the region of 525 millimicrons. The blank used for this determination is a mixture of equal volumes of absolute methyl alcohol and the picric acid reagent.

To establish the value obtained in the tablet assay we have prepared a standard transmission-concentration curve for digitoxin. In this connection we are indebted to Dr. F. O. Laquer of Temple University for his kind cooperation in supplying us with a sample of the digitoxin which he is using in the preparation of the proposed monograph for the Pharmacopoeia. We prepared solutions of this material in absolute methyl alcohol in three concentrations, namely: 2.5 mg., 5.0 mg., and 7.5 mg. per 100 cc. The transmission of each of these solutions was determined 30 minutes after mixing with an equal volume of picric acid reagent in the same manner as described in the tablet assay. The standard curve is thus prepared by plotting the three points in terms of concentration against the logarithm of the transmission and drawing the smooth curve through these points. A typical curve is shown in figure 1.

Under the conditions of the tablet assay the expected digitoxin concentration in the absolute methyl alcohol extract is 5 mg. per 100 cc. By referring the transmission value obtained in the tablet assay to the standard digitoxin curve the assay value can be readily determined.

We have had the kind cooperation of several commercial firms who have supplied us with samples of digitoxin tablets which they manufacture and also samples of the digitoxin used in compounding the tablets. Transmission-con-

centration curves prepared from these samples of digitoxin all showed good agreement with that obtained from the digitoxin sample which we accepted as our standard.

In testing the validity of the assay method we have relied upon a statistical approach in which numerous assays were made on each variety of tablets available. Furthermore, we have varied the number of tablets used for the assay. For example, in assaying 0.2 mg. tablets we have carried out the procedure using 9 and 11 tablets in addition to the prescribed 10 tablets. These results consistently indicated that the values obtained for the 9 and 11 tablet assays were definitely beyond the limits of variation of the value for the 10 tablet assay. Similarly, in the case of the 0.1 mg. tablets we have carried out the assay using

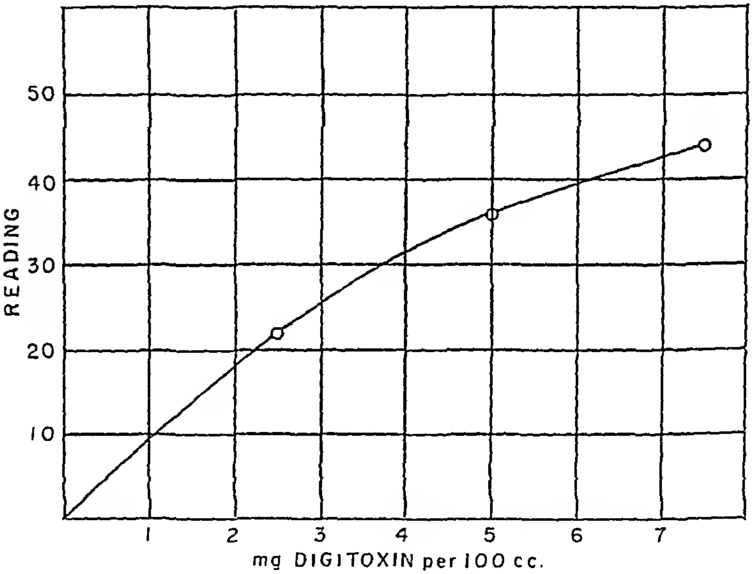

FIG. 1

18, 19, 21, and 22 tablets in addition to the prescribed 20 tablets. Values obtained for 19 and 21 tablet assays frequently fell within the limit of variation of those obtained for the 20 tablet assays while 18 and 22 tablet assay values consistently and definitely fell beyond these limits. These results indicated that the assay method is accurate within a limit of ±5%. Our data also indicate a high degree of uniformity of digitoxin content in each of the varieties of tablets examined.

In table 1 we list typical data obtained in the assay of tablet A which contained 0.1 mg. of digitoxin per tablet. The assay values have been determined by referring the observed readings to the standard curve (figure 1) as previously

described. The calculated values are based on perfect uniformity of the tablets with respect to their digitoxin content.

We encountered two failures in applying the assay method. In the case of one variety of tablets a slight but definite turbidity appeared shortly after the picric acid reagent was mixed with the methyl alcohol extract. On longer standing a slight flocculent precipitate was visible. In this case it seems probable that the absolute methyl alcohol has extracted one of the ingredients of the excipient

TABLE 1

NO. OF TABLETS	READING (LOG OF TRANSMISSION)		MG. DIGITOXIN PER 100 CC.	
			Calc.	Found
18	34.5 33.5 34.5 33.0	33.9	4.50	4.6
19	36.0 35.0 35.0 35.0	35.3	4.75	4.9
20	37.0 35.5 37.0 36.5 36.0 36.5 36.5 36.0	36.2	5.00	5.1
21	38.0 37.5 36.5 38.0	37.5	5.25	5.4
22	38.5 38.0 38.0 38.5	38.3	5.50	5.6

which is subsequently precipitated when the picric acid reagent, containing considerable water, is added. Since this difficulty was encountered in only one instance it appears that the composition of the excipient can be readily altered so as to avoid this complication. In this connection it is to be noted that the use of lactose, one of the favorite excipients, is permissible since its solubility in absolute methyl alcohol is of sufficiently low order.

The other case of failure of the assay involved a variety of tablets which are red in color. The colored substance was soluble in absolute methyl alcohol and

was therefore extracted from the tablet along with the digitoxin. Transmission measurements of the final solution were, of course, completely erroneous. It should be observed that the use of coloring substances, which the manufacturer may find desirable, is not prohibited by the assay method. The requirements are that such a substance must be chemically inert under the conditions of the assay and that its solutions do not show any appreciable absorption in the optical region in which the Baljet reaction appears. In general therefore, red coloring substances are prohibited.

Dr. P. W. Wilcox of Sharp and Dohme has applied our method of assay to three lots of digitoxin tablets. In a private communication he has kindly reported the following results including bioassay values.

	Colorimetric	*Bio-assay*
Lot No. 1	100 per cent 101 per cent	101.6 per cent
Lot No. 2	101 per cent	113 per cent
Lot No. 3	98 per cent 98 per cent	92.5 per cent

SUMMARY

1. A colorimetric method for the assay of tablets of digitoxin containing 0.1 mg. and 0.2 mg. of digitoxin per tablet has been described.

2. The method, based on the Baljet color reaction and requiring a photoelectric colorimeter, is accurate within ±5 per cent.

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CHEMOTHERAPY OF PLASMODIUM KNOWLESI INFECTIONS IN MACACA MULATTA MONKEYS[1]

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Plasmodium knowlesi was first described as a distinct species of *plasmodium* by Sinton and Mulligan (11). Other investigators (Franchini, Napier and Campbell (5), Knowles and Das Gupta (6)) had described what is believed to be the same parasite somewhat earlier, but were dealing with mixed infections and did not attempt to give the parasite a specific name. In all cases the original host of *Plasmodium knowlesi* appears to be *Macacus* (Silenus) *irus*, an oriental monkey, which is usually also infected with another species of malaria parasite, *Plasmodium inui*. When *Macaca mulatta* monkeys,[2] the common monkey of Northern India, are inoculated from *M. irus* monkeys found infected in nature and showing mixed infections, the resultant infection in the majority of cases is one in which only *P. knowlesi* can be detected. This parasite is quite benign in *Macacus irus* monkeys but produces high degrees of parasitemia in *M. mulatta*, generally resulting in death. The apparently pure strains of *P. knowlesi* which we now have were isolated by repeated subinoculations in *M. mulatta* monkeys. Large numbers of *M. mulatta* monkeys have been examined by several English workers in India, and this monkey has thus far not been found to be infected with any malaria parasite in nature.

After *P. knowlesi* was established in laboratory colonies of *M. mulatta*, a great deal of experimental work was initiated, not only with regard to chemotherapy but in other fields of investigation as well. Unfortunately, however, as is generally the case when new host-parasite relationships are discovered, no attempt was made to follow standard techniques for inoculation. The fact that highly fatal infections could be produced with *P. knowlesi* infections in *Macacus mulatta* regardless of the inoculating dose of parasites or the site of inoculation was apparently all that was desired. Some of the earlier work on new drugs was carried out with *P. knowlesi* infections in *Macacus radiatus*, and comparatively benign infections are produced in this host. Some attempts have been made to determine the effect of quinine, atabrine, and plasmochin on *P. knowlesi*, but the widely different dosage schedules used, together with various routes of ad-

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Tennessee. The data was collected and prepared for publication under a contract between the Office of Scientific Research and Development and the Squibb Institute for Medical Research.

[2] We have used the terminology adopted by Ruch "Bibliography of Primate Malaria" N.R.C. 1941. According to this system *Macacus rhesus* is synonymous with *Macaca mulatta*.

ministration employed and different times in the course of the disease when treatment was administered, make it almost impossible to obtain from previous work a clear-cut, over-all picture of the effects of these drugs on this infection.

VARIATIONS IN THE COURSE OF INFECTIONS IN UNTREATED MONKEYS. The strain of *Plasmodium knowlesi* used by us was obtained from Dr. G. Robert Coatney of the National Institute of Health. Intravenous inoculation with parasites was used throughout, and a known dosage of parasites per kilogram body weight was employed. Various doses were employed at first in order to determine the number of parasites which would produce the most satisfactory infections for testing drugs. Doses ranging from 10,000 to 1 billion parasitized cells per kilogram were tried and 50 million parasitized cells per kilogram were selected for routine work in chemotherapy, because it permitted about the same time interval for treatment as used in avian malaria screening procedures. Monkeys inoculated intravenously with this dosage of parasites generally showed about 0.05 to 0.1 per cent parasitized cells in the peripheral blood on the day after inoculation and the degree of parasitemia then increased from day to day until in most animals from 30 to 95 per cent of the cells were parasitized within 5 or 6 days. Death usually occurred in untreated infections on the 6th or 7th day after inoculation following the above dosage.

In table 1 daily parasite counts are given on 46 untreated monkeys inoculated intravenously with 50 million parasitized cells per kilogram. It will be noted particularly that considerable variation occurred in the rate of increase of parasitemia, although a general trend usually occurred (fig. 1). It was rarely until the 2nd or 3rd day after inoculation that the parasite count began to rise above the level of a few scattered parasites in the thin film. On the 4th or 5th day after inoculation most of the monkeys for which data are given in table 1 showed parasite counts over 30 per cent, and death on the day following a count of from 30 to 90 per cent was the rule. It should be noted here that the parasite counts given in the table were all taken at approximately the same time each day (8:00 A.M.–9:00 A.M) when most of the parasites were ameboids or presegmenters. Since *P. knowlesi* has a 24 hour asexual cycle and segmentation usually occurs in the late evening (8:00 P.M.–12:00 M), in most of our animals at least, counts made about 10:00 P.M. were in general much higher than counts made in the morning (fig. 2). This accounts in some instances for the low parasite counts on the day preceding death, since the morning counts only are recorded in the table. As illustrated in figure 2, a count of 5.3 per cent parasitized cells made in the morning may increase to 31 per cent at 10:00 P.M. on the same day during segmentation.

Four of the monkeys given in table 1 survived the infection and became chronic without treatment. This is a somewhat higher percentage of survivals than previously reported. Wats and Harbhagwan (12), for example, state that of 254 *Macacus rhesus* monkeys infected experimentally with *P. knowlesi* in India only 4 did not die, a survival rate of approximately 1.5 per cent. Whether or not the infections in all of these animals were allowed to continue until death occurred without interference of any kind is not stated, however. Krishman,

TABLE 1

Untreated Plasmodium knowlesi infections in Macaca mulatta monkeys inoculated intravenously with 50 million parasites per kilogram

MONKEY NO.	PERCENTAGE PARASITIZED CELLS ON DAYS AFTER INOCULATION												REMARKS
	1	2	3	4	5	6	7	8	9	10	11	12	
440		(−)		0.6	6.5	47.0	D						
441		(−)		0.2	5.6	51.0	D						
443		(−)	0.4	4.6	35.0	D							
449	(−)	0.1	2.3	12.6	12.4	40.0	D						
461	(−)	0.2	1.5	6.3	42.2	38.0	D						
462	(−)	0.3	1.4	13.6	38.8	93.5	D						
489	(−)	0.2	3.1	8.0	27.0	65.0	D						
490	(−)	0.6	2.9	24.0	73.0	D							
503	0.3	2.4	7.1	34.0	48.0	D							
504	(−)	0.9	3.4	11.0	32.0	14.2	21.0	63.0	D				
516	(−)	(−)	0.4	0.9	6.8	6.5	10.4	8.3	18.0	9.4	2.3		Chronic
517	(−)	(−)	0.6	2.9	9.6	72.0	D						
518	0.4	0.3	1.8	31.8	D								
519	(−)	0.3	1.7	4.0	7.5	1.8	(−)	(−)	(−)	(−)			Chronic
522	(−)	(−)	0.5	4.0	13.0	D							
537		2.1	3.6	8.0	17.0	29.0	18.5	5.2	2.3	(−)		0.5	Chronic
538		0.5	2.4	22.0	37.5	D							
539		0.7	4.8	16.3	29.0	88.0	D						
540		0.8	2.1	21.0	48 0	14 3	34.0	D					
541	(−)	0.5	3.0	12.5	D								
553	(−)	(−)	0.6	3.0	7.1	19.0	D	D					
554	0.3	0.7	13.6	76.0	D								
556		0.2	0.1	3.1	34.0	D							
607	(−)	0.6	1.6	5.1	4.2	10.2	4.5	1.6		0.3	0.1		Chronic
608	(−)	0.1	0.5	6.7	28.0	43.0	D						
614		0.2	1.2	26.2	71.0	D							
615		0.3	0.5	16.0	10.8	20.6	60.0	64.0	D				
629		1.9	3.0	39.0	D								
654	(−)	0.5	4.6	34.0	72.0	D							
655	(−)	1.1	1.9	22.0	70.0	D							
656	(−)	0.8	2.9	15.8	31.2	26.0	12.0	7.2		23.0	D		
657	(−)	1.0		28.0	D								
669	(−)	(−)	0.6	2.3	12.0	38.0		8.2		D			
670	(−)	(−)	0.3	8.1	12.4	D							
709		0.1	0.3	14.4	23.2	D							
717		0.2	8.4	23.0	D								
718		0.1	0.4	4.8	70.0	D							
719		4.1		84.0	D								
720		2.1		86.0	D								
731		0.4	1.0	6.5	39.0	D							
732		0.3	1.4	14.0	66.0	D							
754		0.7	2.2	24.0	D								
755		0.6	6.0	20.0	D								
777		0.6	12.5	60.0	D								
778		0.6	6.6	44.0	D								
800		0.3	7.2	26.4	D								

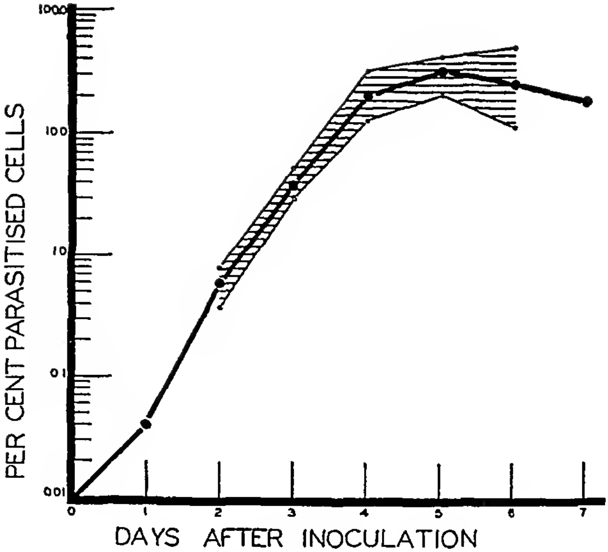


FIG. 1. COURSE OF UNTREATED PLASMODIUM KNOWLESI INFECTIONS IN 46 MACACA MULATTA MONKEYS

Inoculated intravenously with 50 million parasitized cells per kilo. Shaded areas represent the mean parasite count ± two standard errors

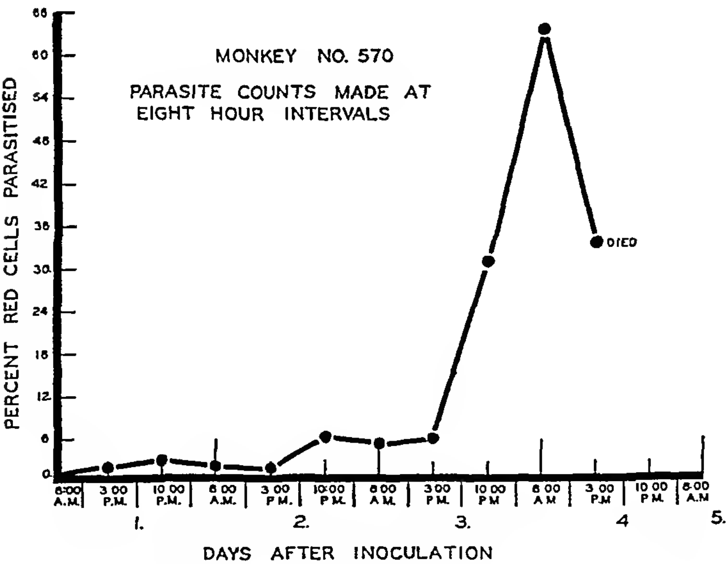


FIG. 2. COURSE OF PARASITEMIA IN A MONKEY GIVEN 50 MILLION PARASITES PER KILO INTRAVENOUSLY

Parasite counts made at 8 hour intervals. Note marked increase in parasites between 3:00 P.M. and 10:00 P.M. on third day

Lal and Napier (7) found 1 monkey to be refractory to infection out of 12 which they inoculated. Another monkey out of the eleven which became infected showed a tendency toward natural cure and survived for 32 days without treatment. Coggeshall and Kumm (13) report only 1 survival in 70 infections. *Plasmodium knowlesi* is therefore not invariably fatal to *M. rhesus* monkeys, but for all practical purposes death can be expected to occur if treatment is not given.

Figure 3 summarizes data from untreated infections which may be of value in judging the effect of drugs on this host-parasite relationship. All of these animals were infected with the standard intravenous dosage of 50 million parasitized cells per kilogram. In figure 3 (b) the blocks represent number of animals dying on days after inoculation. No deaths were observed before the fifth day. However, by the seventh day 80.2 per cent of the animals had died. In this group of 46 monkeys all those which survived eleven days after inoculation lived for an indefinite period of time. The average day of death for 42 out of 46 animals which died was 6.3 ± 0.71.[3]

Because of the rapid increase in parasite count from day to day, it was impossible to select any one day which would be the parasite peak for all animals. However, if one considers the day on which peak of parasitemia was reached, as is illustrated in figure 3 (a) it is seen that the vast majority of the animals had their highest parasite count by the sixth day after inoculation and regardless of whether they died or not all animals reached their peak of parasitemia before the tenth day.

From this analysis of data on untreated infections we selected three observations to be used as criteria of the effect of drugs:

a) *Per cent mortality.* As pointed out above, survival cannot be considered an absolute criteria of activity of a drug when a small number of animals are used. However, death of an animal within ten days after inoculation must be considered as presumptive evidence that either no antimalarial effect was exerted or that the drug was toxic.

b) *The day of death.* In cases in which a drug exerted a slight or suppressive action and in which relapse occurred, per cent mortality may have been just as great as in the untreated controls. In such instances, however, this suppressive action would be reflected in a prolongation of the mean day of death.

c) *Highest parasite count.* Since all untreated animals reached a peak of parasitemia by the ninth day, we arbitrarily selected for each animal the highest of the daily counts observed up to and including the tenth day and have used this for comparison with untreated controls. In 46 untreated animals the mean highest parasite count was 45.4 ± 3.54.[3] In only one animal was a parasite peak below 10 per cent observed.

Methods used for chemotherapeutic studies. All animals were inoculated intravenously with a standard dose of 50 million parasitized cells per kilogram. Donor blood was taken from an untreated, infected animal with a parasite count of 20 to 50 per cent. Each experimental animal was placed in an

[3] Mean plus or minus standard error.

individual cage and fed a liberal diet of milk, eggs, bread, fresh vegetables, fruit and peanuts. Blood for parasite counts were taken daily at approximately the same hour (8:00 A.M.–9:00 A.M.)

All drugs were administered by stomach tube in a volume of 10 cc. per monkey for each dose. Soluble drugs were dissolved in water and insoluble ones were

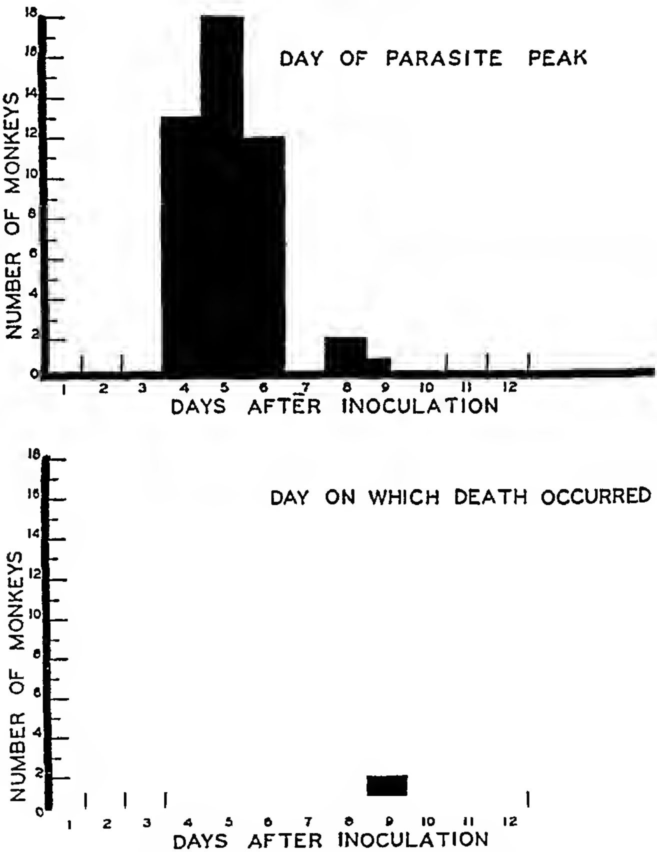


FIG. 3. COURSE OF UNTREATED PLASMODIUM KNOWLESI INFECTIONS IN MACACA MULATTA MONKEYS FOLLOWING INTRAVENOUS INJECTION OF 50 MILLION PARASITIZED CELLS PER KILO

suspended in a 2.5 per cent aqueous starch emulsion. In order to maintain a reasonably constant blood level, each daily dose was given in three divided doses at approximately 8-hour intervals (8:00 A.M; 3:00 P.M.; and 10:00 P.M.)

Because of limitation of cage space, it was ordinarily possible to run only 10 or 15 animals at any one time. In order to utilize the limited number of monkeys to the best advantage it was necessary to use only a few doses of any one com-

pound in any one experiment, and to await the results of each series of tests before planning additional experiments. Because of this plan the final results of a number of experimental series, carried out at different times, had to be combined and compared. Table 2 illustrates one such typical experiment.

THE EFFECT OF QUININE ON PLASMODIUM KNOWLESI INFECTIONS. As noted previously, several investigators have published data on the effect of quinine, as well as other antimalarial drugs on *P. knowlesi*. Row, Dalal and Gollor Kori (10) state that a dose of 2.5 grains of quinine checked the infection but a recrudescence occurred within a few days which was fatal unless treated. Chopra and Das Gupta (1) found the immediate response to quinine slower than to

TABLE 2

Chemotherapy P. knowlesi in Macaca mulatta monkeys

Protocol of experiment: 8-7-41—Inoculated with 50 million parasites per kilo. All drugs administered by stomach tube three times daily at eight hour intervals. Volume of each dose 10 cc. Treatment begun 18 hours after inoculation and continued for 5 days.

MONKEY NUMBER	DRUG	TOTAL DAILY DOSE	DAYS AFTER INOCULATION									REMARKS
			2	3	4	5	6	7	8	9	10	
			Per cent red cells parasitized									
		mgm/kgm										
736	Sulfadiazine	0.03	0.9	3.0	12.0	2.0	2.6	0.4	14.4	48.0	D	
737	Sulfadiazine	0.03	0.2	(+)	0.1	0.1	4.2	7.4	61.6	70.0	D	
738	Sulfadiazine	0.06	0.3	4.0	1.4	(+)	2.0	7.2	40.0	35.6	D	
739	Sulfadiazine	0.06	0.1	(−)	(−)	(+)	(+)	(+)	0.2	0.3	3.0	Chronic
740	Sulfadiazine	0.12	0.3	0.2	1.0	0.25	0.5	1.5	4.4	3.1	0.8	Chronic
741	Sulfadiazine	0.12	0.1	0.7	1.0	(+)	0.2	3.5	2.6	20.4	4.0	Chronic
742	Sulfadiazine	0.24	(+)	0.2	(+)	(+)	(+)	(+)	0.3	77.2	32.0	Died
743	Sulfadiazine	0.24	0.2	(+)	(+)	(+)	(+)	0.1	0.3	1.7	2.4	Chronic
747	Quinine bisulf.	18.75	0.4	1.0	22.0	26.0	D					
748	Quinine bisulf.	37.50	0.3	4.0	7.0	0.7	(+)	(+)	(+)	8.0	23.4	
753	Sulfadiazine	1.0	0.1	5.8	58.0	D						
	PABA*	30.0										
754	Untreated		0.7	2.2	21.0	D						
755	Untreated		0.6	6.0	20.0	D						

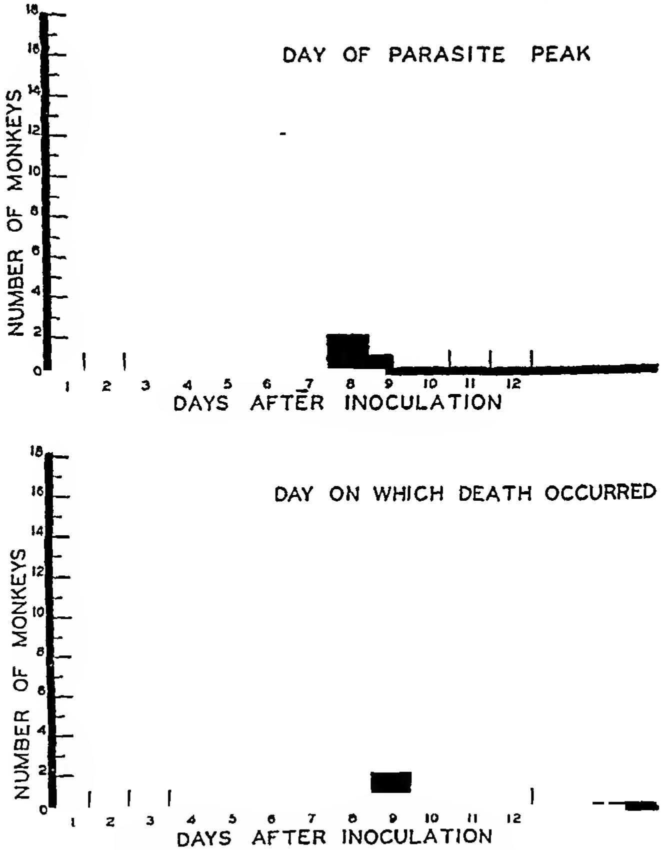
* Para amino benzoic acid.

atabrine. Chopra, Ganguli and Roy (2) were unable to save monkeys with high doses of quinine when the number of parasites reached 1 million per cmm. Nauck (9) found that the effective dose of quinine was higher than in human malaria and that quinine acted slowly on the dividing forms of the parasites. In these and other reports, however, the method of drug administration, the number of doses of quinine and the time during infection when treatment was started were not comparable. Moreover, a standard parasite inoculum was not used. It was futile, therefore, to attempt to compare the results obtained by different investigators under these conditions or to evaluate the results in terms of other experimental infections.

Table 3 summarizes data on 31 monkeys given various doses of quinine bi-

individual cage and fed a liberal diet of milk, eggs, bread, fresh vegetables, fruit and peanuts. Blood for parasite counts were taken daily at approximately the same hour (8:00 A.M.–9:00 A.M.)

All drugs were administered by stomach tube in a volume of 10 cc. per monkey for each dose. Soluble drugs were dissolved in water and insoluble ones were



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Protocol of experiment: 8-7-44—Inoculated with 50 million parasites per kilo. All drugs administered by stomach tube three times daily at eight hour intervals. Volume of each dose 10 cc. Treatment begun 18 hours after inoculation and continued for 5 days.

MONKEY NUMBER	DRUG	TOTAL DAILY DOSE	DAYS AFTER INOCULATION — Per cent red cells parasitized: 2	3	4	5	6	7	8	9	10	REMARKS
		mgm./kgm										
736	Sulfadiazine	0.03	0.9	3.0	12.0	2.0	2.6	0.4	14.4	48.0	D	
737	Sulfadiazine	0.03	0.2	(+)	0.1	0.1	4.2	7.4	61.6	70.0	D	
738	Sulfadiazine	0.06	0.3	4.0	1.4	(+)	2.0	7.2	40.0	35.6	D	
739	Sulfadiazine	0.06	0.1	(−)	(−)	(+)	(+)	(+)	0.2	0.3	3.0	Chronic
740	Sulfadiazine	0.12	0.3	0.2	1.0	0.25	0.5	1.5	4.4	3.1	0.8	Chronic
741	Sulfadiazine	0.12	0.1	0.7	1.0	(+)	0.2	3.5	2.6	20.4	4.0	Chronic
742	Sulfadiazine	0.24	(+)	0.2	(+)	(+)	(+)	(+)	0.3	77.2	32.0	Died
743	Sulfadiazine	0.24	0.2	(+)	(+)	(+)	(+)	0.1	0.3	1.7	2.4	Chronic
747	Quinine bisulf.	18.75	0.4	1.9	22.0	26.0	D					
748	Quinine bisulf.	37.50	0.3	4.0	7.0	0.7	(+)	(+)	(+)	8.0	23.4	
753	Sulfadiazine	1.0	0.1	5.8	58.0	D						
	PABA*	30.0										
754	Untreated		0.7	2.2	24.0	D						
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Table 3 summarizes data on 31 monkeys given various doses of quinine bi-

sulfate. In 7 animals given up to 18.6 mgm. per kilogram a day, only 2 had a course which indicated an antimalarial effect; however, even these 2 animals relapsed and died as soon as treatment was discontinued. 37.25 mgm. per

TABLE 3

Summary of effect of drugs on P. knowlesi infections in Macaca mulatta monkeys inoculated intravenously with 50 million parasites per kilogram

DOSE OF DRUG	NUMBER OF MONKEYS	MORTALITY		MEAN OF HIGHEST PARASITE COUNTS UP TO AND INCLUDING 10TH DAY*	ESTIMATE OF ACTIVITY	REMARKS
		Per cent	Mean day of death			
			Untreated controls			
mgm. per kilo per day	46	91.3	6.3 +0.71	45.4 +3.5		
			Quinine bisulfate			
18.6 (11.1)†	7	100.0	7.5	41.3		
37.2 (22.2)	10	50.0	13.6	9.5		
75.0 (44.4)	9	22.0		5.3		
150.0 (88.8)	5	0.0		0.5		
			Sulfadiazine			
0.015	6	100.0	7.5	49.2		
0.03	6	100.0	7.6	46.0		
0.06	7	86.0	8.6	34.8		
0.12	7	42.8	20.0	13.0	Q 175.0	
0.24	5	0.0		6.7		(2/2 "Cure")‡
0.50	4	0.0		(+)		(3/3 "Cure")
1.00 and over	19	5.0		(−)		(10/10 "Cure")
			Sulfadiazine plus daily dose of 30 mgm. per kilo of *p-amino benzoic acid*			
1.00	1	100.0	5.0	58.0		
1.50	3	100.0	5.8	61.0		
3.00	2	100.0	5.5	68.0		
6.00	2	100.0	5.5	37.6		
7.50	1	0.0		9.6	Q 1.0±	
12.00	1	100.0	6.0	62.0		
37.50	1	0.0		0.05		
187.50	1	0.0		0.2		

* Per cent of red cells parasitized.

† Figures in parenthesis refer to milligrams of quinine alkaloid.

‡ Successfully reinfected.

kilogram per day produced a variable but significant effect, while 75 mgm. per kilogram per day exerted an antimalarial effect in all animals. Daily doses of 150 mgm. per kilogram had a profound antimalarial effect with survival of all animals for an indefinite period of time, however, at this dosage scattered para-

sites were found for periods up to 2 months after inoculation. Hence it is obvious that sterilization had not occurred.

THE EFFECTS OF SULFADIAZINE ON P. KNOWLESI INFECTIONS. Coggeshall (3, 4) and others have shown that large doses of sulfonamides of various types exert a profound plasmodicidal effect on *P. knowlesi* and that under some conditions sterilization was produced. As far as we can determine, no one has determined the range of doses of any sulfonamide at which this antimalarial effect is produced. Table 3 presents a summary of our attempts to obtain this information. Daily doses up to 0.06 mgm. per kilogram of sulfadiazine produced no consistent effect, but doses of 0.12 mgm. per kilogram produced a definite effect which was comparable to 22.2 mgm. per kilogram of quinine alkaloid indicating that on the basis of dosage by mouth sulfadiazine is 175 times as effective as quinine.

On a dose of 0.24 mgm. per kilogram of sulfadiazine two of five monkeys showed no parasites in blood films following treatment and on reinoculation these animals developed typical infections. Although such a reaction is not absolute, it is presumptive evidence that these monkeys had been sterilized with sulfadiazine. On still higher doses of sulfadiazine the number of monkeys showing no relapse after treatment was greater. Representative animals of each group which were reinoculated all developed typical infections.

The dose of sulfadiazine needed to treat *P. knowlesi* is so small that all attempts at relating this effect to blood level were completely unsuccessful. We believe the procedure for analysis of sulfonamides used by us was accurate down to approximately 0.5 mgm. per cent so that the effective concentration of this agent in monkey blood must have been below this.

Because of the evidence that para-aminobenzoic acid is effective in blocking the antibacterial action of some sulfonamides, we thought it worth while to determine if such an antagonism could be demonstrated for the antimalarial action of sulfadiazine. Accordingly, we attempted to determine the dosage-response curve of sulfadiazine when administered in conjunction with total daily doses of para-aminobenzoic acid, 30 mgs. per kilogram in three divided doses. The result of these experiments are also presented in table 3. The number of animals used is insufficient to allow detailed conclusions, but it is probable that the minimal effective dose of sulfadiazine when combined with p-aminobenzoic acid is somewhere between 6 and 37.5 mgm. per kilogram per day. It seems quite certain that at least 100 times as much sulfadiazine is required in the presence of this dose of para-aminobenzoic acid to produce an antimalarial effect as in the absence of it.

THE EFFECT OF DELAYED TREATMENT ON THE ACTION OF QUININE AND OF SULFADIAZINE. Other studies in man and in animals have indicated that sulfadiazine acts more effectively as a suppressive agent than as a curative agent. Therefore, we thought it important to see whether the striking action of sulfadiazine reported above was due to the fact that treatment was begun early in the disease at a time when only a few parasites were present and was a "suppressive" rather than a "therapeutic" action. In order to test this, experiments

were carried out in which instead of beginning treatment fifteen hours after inoculation treatment was delayed until 72–80 hours after inoculation. Table 4 illustrates the effect of quinine and of sulfadiazine when used in this way.

At the time of beginning treatment parasites counts in the experimental groups were as high or higher than those ordinarily seen in untreated controls. For approximately 24 hours the course of the disease in treated animals was

TABLE 4

Effect of delayed treatment of P knowlesi with quinine bisulfate and sulfadiazine

Macaca mulatta monkeys inoculated with 50 million parasitized cells per kilo—treatment started 72 hours after inoculation, three daily doses at 8 hour intervals

MONKEY NO	TOTAL DAILY DOSE	PER CENT PARASITIZED CELLS ON DAYS AFTER INOCULATION									
		2	3	4	5	6	7	8	9	10	11
		Quinine bisulfate									
	mgm /kgm										
769	18.7*	0 05	7 0	D							
795	18 7	0 25	6 2	33 6	D						
770	37 5	0 8	10 4	37 2	30 4	0 3	(+)	0 05	(−)	0 3	
796	37.5	(+)	4.2	24 2	D						
749	37 5	1 2	17 2	38 0	39 0	(+)	(+)	(+)	(−)	(−)	
750	37 5	0 6	16.0	34 0	14 8		0 05	(+)	0 1	(−)	
751	75 0	0 7	18 0	46 0	1 5	0 5	0 05	(+)	(+)	(−)	
752	75 0	0 4	8 0	19 0	1 5	(+)	(+)	(−)	(−)	(−)	
		Sulfadiazine									
792	0 06	0 1	4 2	26 0	34 4	D					
764	0 12	0 8	2.6	42 4	0 2	0 1	(+)	(−)	(−)		
765	0 24	0 6	5 8	35 6	8 0	0 4	0 1	(+)	(+)	2 0	
766	0 48	0 3	1 9	51 0	6 4	0 2	(+)	(+)	(−)		
744	0 48	0 4	2 0	10 0	0 1	(+)	(+)	0 2	(−)	(−)	(+)
745	0 96	0 6	2 8	27 0	7 2	(+)	(+)	(+)	(−)	(−)	
746	1 92	1 2	7 2	25 0	(+)	(−)	(+)	(+)	(−)	(+)	
		Untreated controls—mean of 46 monkeys									
		0 6	2 8	20 2	31 5	27 8		(80 2% dead by 7th day)			

* In terms of bisulfate; to obtain dosage of quinine base multiply by 0 6

similar to untreated animals, and the parasite peak reached was equally high in both groups. In spite of this, animals given an adequate dose of quinine or sulfadiazine improved and survived whereas most of the untreated controls died. It is of interest that the effective dose of these two drugs under these conditions was about the same as when treatment was started within fifteen hours after inoculation

CONCLUSIONS

1. Detailed studies of the course of untreated *P. knowlesi* infections in *Macaca mulatta* monkeys have indicated how this host-parasite relationship may be used for quantitative chemotherapeutic studies.

2. Sulfadiazine is approximately 175 times as active as quinine in the treatment of this experimental infection.

3. It has been demonstrated that the chemotherapeutic effect of sulfadiazine *in vivo* in monkeys can be antagonized by para-aminobenzoic acid.

4. Delaying treatment of *P. knowlesi* infections in monkeys until the disease has become well established has no effect upon therapeutic effects of quinine or sulfadiazine. Both appear to act with equal rapidity.

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AGGREGATION AS A FACTOR INFLUENCING THE TOXICITY OF SYMPATHOMIMETIC AMINES IN MICE

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INTRODUCTION. In the course of a pharmacological study, involving the measurement of analeptic activity (1), it was necessary to determine the toxicity of picrotoxin and amphetamine. No difficulty was encountered in determining the L.D.50 of picrotoxin; the values found showed excellent agreement with those already recorded. When amphetamine was examined, an entirely different situation arose. No consistent results could be obtained.

Gunn and Gurd (2) found, with benzedrine and allied compounds, "which produce a similar stimulation of the central nervous system, that the symptoms of excitement are much more pronounced if several injected animals are kept together in a cage or tray. If one animal is kept alone in a jar, no very striking symptoms of excitement may be exhibited, whereas when several are together and especially if they have room to run, they excite one another". This excitement was very conspicuous in mice receiving toxic doses of amphetamine in this laboratory. From this it seemed probable that the presence of other mice confined within a limited space might be a factor influencing the toxicity of this drug.

Aggregation of animals in cages is known to affect a number of animal characteristics (3). It was, therefore, thought worth while to discover whether confining a number of mice together influenced the toxicity of amphetamine and related compounds for mice. Ephedrine and methedrine were also studied because of their known stimulation of the central nervous system and adrenaline was included because of its close chemical relationship to the other amines associated with a much less pronounced action on the central nervous system.

METHODS. Large numbers of mice were required for the investigation, as it was decided to make each set of observations on a single occasion in order to eliminate uncontrolled factors which might influence the toxicity of these drugs. The mice were obtained from outside sources on two occasions; on the third, tests were made with a healthy stock of fawn (GFF) animals extracted by brother-sister mating for five to ten generations from the "farm stock" supplied by the Agricultural Research Council Field Station, Compton.

In each test the dose was administered in the same volume of water. The room temperature was maintained at 80°F. Only animals of one sex were used on any one occasion. The body weights of the mice were confined within the limits of 18 to 22 grams. Dosing was in proportion to body weight.

The hydrochlorides of adrenaline, methedrine and ephedrine and the sulphate of amphetamine, have been used for this work. All of these were dissolved in redistilled water and single injections were made with a number 14 "Record" needle into the subcutaneous space behind the head.

At least two responses were obtained on the dosage mortality curve close to the median response, with a minimum of ten mice at each dose level. The regression co-efficient of probit percentage mortality on log dose; the L.D. 50 were calculated by the method of least squares and the values were checked by plotting the equation to the line found by calculation. Weighted values were obtained for the probits whenever the distribution of the response was unsymmetrical about probit 5.0 or when 0% or 100% responses were included in the calculations. In calculating the values of L.D. 50 and of b (regression coefficient), we have followed substantially the method of Bliss (1938). The values of s^2 and s^2_b consequently represent the variances of a single observation and of the regression coefficient respectively. They are the squares of the corresponding logarithmic standard

TABLE 1

*Effect of social environment on toxicity**

SUBSTANCE	L.D.50 (MGM./KGM.)			REGRESSION CO-EFFICIENT (b)		
	1 in box	10 in box	Mean ratio	1 in box	10 in box	Mean ratio
Amphetamine						
1st test	117.3 (0.001301)	14.01 (0.004606)	9.95	7.18 (1.098)	5.08 (7.238)	0.72
2nd test	89.55 (0.001221)	7.00 (0.00433)		8.56 (6.885)	5.09 (3.9)	
Methedrine	23.26 (0.00002078)	7.56 (0.002028)	3.09	3.53 (0.003996)	8.57 (1.983)	2.41
Ephedrine						
1st test..	100.0 (0.01546)	43.92 (0.01856)	4.00	4.26 (5.020)	4.89 (7.841)	0.99
2nd test	238.58 (0.004275)	40.87 (0.006770)		3.00 (0.6373)	2.27 (0.05374)	
Adrenaline						
1st test	3.96 (0.003213)	2.17 (0.00491)	2.06	5.59 (5.6)	4.45 (4.1)	0.53
2nd test	4.58 (0.008164)	1.98 (0.00909)		6.00 (16.75)	1.68 (3.6)	

Figures for logarithmic variance, s^2, are given in parentheses.

* GFF mice, ♂. See p. 214

errors. The limits of error for any value of L.D.50 are found by multiplying s by the appropriate factor (e.g. 1.96 for P = 0.95).

RESULTS. (*a*) *The effect of the presence of other mice.* The effect of the presence of other mice is shown in table 1. It is seen that if the area per mouse is kept constant, the presence of nine other mice doubles the toxicity of adrenaline and increases that of amphetamine nearly ten times. The effect on the toxicity of ephedrine and methedrine lies between that of these two substances. The results grade the substances in a manner which parallels their relative

stimulation of the central nervous system and may be an expression of this activity.

(b) *Effect of central nervous excitation on behaviour.* Observation of the mice is essential to any understanding of the exceptional sensitivity to the presence of other mice which these drugs induce in a single mouse. The central nervous stimulation produced by amphetamine, ephedrine and methedrine changes the behaviour of the mice so that they begin moving rapidly round the cage, running over and under each other just as they would normally avoid other obstacles in

1a

1b

1c

1d

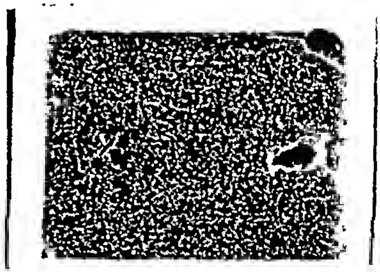

PLATE I

1a. Group of normal mice.

1b. Excessive running of "Benzedrinised" mice at early stage in social activation.

1c. "Defensive encounters" between pairs of "Benzedrinised" mice under social activation.

1d. Normal mouse (centre), and "Benzedrinised" mouse (corner) showing typical "apprehensive" posture.

their path (Plate 1b). At the same time the animals assume characteristic postures when not in motion (Plate 1d). The reactivity of the mice is increased by the simultaneous appearance of an "apprehensive attitude" (see below), so that their behaviour becomes related to that of other mice in the box in such a way that mutual excitation of one mouse by another ultimately increases the excited behaviour of the group to tumultuous proportions. Finally, abnormal convulsive behaviour ensues in some mice, leading eventually to their death. Adrenaline, on the other hand, induces merely this postural change, without much pronounced motor stimulation. Convulsions appear suddenly without

prior symptoms in mice receiving adrenaline. They are very violent and almost invariably lethal. Thus, a dose that was initially non-toxic becomes lethal through the alteration of the psychic condition it brings about in the mice.

Reference has already been made to the observation of Gunn and Gurd who have noticed that in groups of mice "the symptoms of intoxication are very characteristic; the animals are extremely restless, make frequent rapid rushes often accompanied by squealing, and sometimes sit up on their hind quarters with inquisitive rotational movements of the head and neck. Occasionally there are clonic convulsive movement." When the individual mouse that has received 10 mgm./kgm. of amphetamine is alone in a box, periodic squeaks and rapid running movements occur spontaneously. The rapid running movements of these mice are interrupted by short periods during which the animal remains rigid and alert, often adopting the characteristic postures mentioned by Gunn. During all these movements it mostly remains close to the sides of the box, with head turned away from the wall, eyes looking upwards and the off hind-legs extended, with a characteristic slinking gait, unlike the normal untreated mouse, which will cross from side to side and often sits cleaning itself fully exposed to view (Plate 1d). In short, therefore, the "benzedrinised" mouse is "apprehensive".

Such mice, when placed together in groups, react to one another's squeaks by little jumps and by adopting a position of readiness for action, made up of the postures just mentioned. If, moreover, they are approached by other mice, they either attempt to run away or adopt a *defensive attitude* towards the approaching animals. In these defensive attitudes the mice rear on their hind quarters with forepaws and noses almost touching. They may remain in this position for as long as a minute, gradually swaying in unison opposite one another and sometimes squeaking. This behaviour we have termed a "defensive encounter", since neither mouse attacks the other, but merely adopts a defensive attitude to it (Plate 1e). Jumping may occur erratically; a mouse may then land near another and in this way evoke a "defensive encounter". On the other hand, the mouse which has been approached and frightened in this way may run away or jump. These are for obvious reasons called "escape reactions". The net result is that in a group of ten "benzedrinised" mice "defensive encounters" and "escape reactions" alternate rapidly. This leads to the most intense activity, which reaches a peak and rapidly declines, leaving some of the mice exhausted or convulsing. Owing to the influence of one mouse upon another, the activity of the group assumes a periodic character. For a moment all the animals will assume rigid postures, then a sound, a squeak or a movement of one of the mice starts violent outbursts of activity to be followed later by another short period of quiet. It is clear, therefore, that a characteristic form of social behaviour is engendered by amphetamine in groups of mice confined within a space sufficiently restricted to bring them into close proximity with each other, but not such that there is insufficient room for each mouse to move independently.

(c) *The effect of varying the degree of aggregation.* This behaviour induced by amphetamine, and to a modified extent by ephedrine and methedrine, suggested

TABLE 2

Effect of area per mouse on mortality due to Ephedrine hydrochloride in ♀ (Swiss) mice, in groups at R.T. 80°F.

Dose: 50 mgm./kgm. in 25 ml. solution. No. of mice per experiment: 32. Box area 590 sq. cm.

EXPT	NO OF GROUPS	NO IN GROUP	AREA PER MOUSE	NO DIED	MORTALITY
			sq. cm		*per cent*
1	1	32	18	30	94
2	2	16	37	18	56
3	4	8	74	11	35
4	8	4	148	0	0
5	16	2	296	0	0

TABLE 3

Effect of area per mouse on mortality due to Amphetamine ("Benzedrine") in ♂ mice,† in groups

Dose: 20 mgm./kgm. body-weight in 20 ml. of solution. No of mice per experiment: 32. Sizes of boxes: 1180, 590, 295 sq. cm.

EXPT.	NO OF GROUPS	NO IN GROUP	AREA OF BOX	AREA PER MOUSE	NO DIED	MORTALITY	MEAN
			sq cm	*sq cm*		*per cent*	
1	2	16	295	18	32	100	100
2	1	32	590	18	31:32*	97:100	99
3	4	8	295	37	22	68	68
4	2	16	590	37	13:25*	41:78	60 } 64
5	1	32	1180	37	21	66	66
6	4	8	590	74	4:12*	13:39	26
7	8	4	590	148	2:0*	6:0	3
8	16	2	590	296	2	6	6

* Duplicate experiments.
† Ex dealer.

TABLE 4

*Effect of temperature on toxicity**

SOCIAL ENVIRONMENT	L.D.50 (MGM./KGM.)		REGRESSION CO-EFFICIENT (b)	
	Room temperature			
	80°F	60°F	80°F	60°F
Solitary	90.0 (0.00141)	197.0 (0.00008683)	10.26 (21.6)	13.18 (2.371)
Groups of 10	13.71 (0.001585)	141.4 (0.005157)	7.88 (5.858)	7.32 (22.56)

Figures for logarithmic variance, s², are given in parentheses
* GFF mice, ♂. See p. 214.

that the effect of aggregating a numbei of mice together might be an expression of the number of encounters between the mice. The toxicity would, then, be a function of the degree of aggregation, as this would alter the frequency of encounters between the mice. This is confirmed by the results presented in tables 2 and 3, which show the effects of varying the area per mouse in a group while simultaneously decreasing the number of mice confined together.

(*d*) *The effect of aggregation at different temperatures.* In an investigation into other factors influencing the toxicity of these amines, the temperature was found to produce an appreciable effect. As, therefore, the amount of activity developed in a box of mice was found to vary considerably from time to time, the influence of temperature on this activity was investigated. It was found that the activity was almost absent at 60°F, but very pronounced at 80°F. The effect of lowering the temperature, moveover, as will be seen in table 4, markedly reduced the effect of aggregation on the toxicity of amphetamine almost to the same figure as that obtained with solitary mice under the same conditions.

SUMMARY

1. Aggregation influences the toxicity of sympathomimetic amines in mice.

2. As the area per mouse increases, the toxicity decreases for mice confined in a group.

3. If the area per mouse is kept constant at approximately 50 sq. cm., the presence of [9] other mice markedly increases the toxicity of these drugs.

4. Environmental temperature greatly influences the degree of mutual excitation and thus modifies the effect of aggregation on toxicity.

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THE ACTION OF TETRAETHYLAMMONIUM ION ON THE MAMMALIAN CIRCULATION

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In a previous paper, the action of tetraethylammonium ion on the heart-lung preparation of the dog was reported (1). It was shown that this substance has a positive inotropic effect on the failing heart not unlike that of veratrum alkaloids or cardiac glycosides. In this paper the action of tetraethylammonium ion on the circulatory system of the intact, anesthetized mammal is described and analyzed. The vasodepressor effect of this substance, which is the main concern of the paper, was observed by Marshall (13) and by Trendelenburg (17). It has hitherto not been subjected to experimental analysis.

The effects which this substance has on the heart rate and arterial pressure are not unlike those produced by veratrum alkaloids (see 9). The mechanism of these actions of tetraethylammonium ion, however, is shown to be quite different from that of veratrum alkaloids. It depends upon an effect similar to the "nicotine paralyzing" action of tetraethylammonium ion which was described by Burn and Dale (3) and later studied with respect to a series of triethylalkyl-ammonium compounds by Hunt (4, 5, et al.).

METHODS. Cats and dogs were used. The anesthetic was usually Dial solution[1] (each cc. containing diallyl barbituric acid [Dial], 0.1 gram, urethane, 0.4 gram, and monoethyl urea, 0.4 gram, in aqueous solution) injected intraperitoneally in a dose of 0.7 cc. per kgm. In a few of the cats and most of the dogs, anesthesia was produced with sodium pentobarbital,[2] injected intraperitoneally in doses of 35 mgm. per kgm. When decerebration or other destruction of the central nervous system was performed, the operation was done under ether anesthesia and unless otherwise noted, the experimental observations were begun one hour after the discontinuation of etherization.

Arterial pressure was recorded with a mercury manometer. Heart rates were counted from the apical impulse or from the electrocardiogram recorded by an inkwriting oscillograph after amplification. Stimuli were applied to nerve trunks through shielded, platinum electrodes from a stimulator delivering square waves or diphasic shocks of known duration at frequencies from 0.5 to 500 per sec. This stimulator was designed and built by Albert M. Grass.

Tetraethylammonium bromide (Eastman Kodak Co.) was used in all experiments and made up in a stock solution containing 100 mgm. per cc and diluted for each experiment. Doses are expressed in terms of the bromide. Elementary analysis and tests for ash and amines showed that the sample of tetraethylammonium bromide used was pure.[3] Intravenous injections were made into a cannula in the femoral or jugular vein and washed into the venous system with 1.5 to 3 cc. of saline. Crude South American curare or Intocostrin[4]

[1] Generously supplied by Ciba Pharmaceutical Products, Summit, New Jersey.

[2] Sodium pentobarbital (Nembutal) was generously supplied by the Abbott Laboratories, North Chicago, Illinois.

[3] Tests done by Dr. Carl Tiedeke, New York.

[4] Generously supplied by E. R. Squibb and Sons, New York City.

was used in certain experiments to paralyze the skeletal muscles. Blood flow in the femoral artery of the dog's leg was recorded by a flow cannula and a differential manometer described by Moe, Bassett, and Krayer (14).

For the exposure of the fourth Ventricle in cats under dial anesthesia, the animal was placed prone on a board and its head was held in a Czermak clamp at such an angle that the floor of the fourth ventricle was horizontal and the neck bent sharply ventrad at the foramen magnum. Through a nuchal incision, the junction of spinal cord and medulla was exposed in a bloodless field. The foramen magnum was then enlarged by removing the middle part of the occipital bone by means of rongeurs. By blunt dissection, the cerebellum was lifted from the fourth ventricle; it was held up by a retractor attached to the top of the cranium. Before each application of tetraethylammonium bromide to the fourth ventricle, the cup-like floor was gently dried by the application of a tiny piece of dry cotton. The substance was then applied in a volume of 0 02 cc.

Results. *I. Arterial pressure and heart rate in the intact animal.* Single intravenous injections of tetraehtylammonium bromide in a dose of from 0.1 to

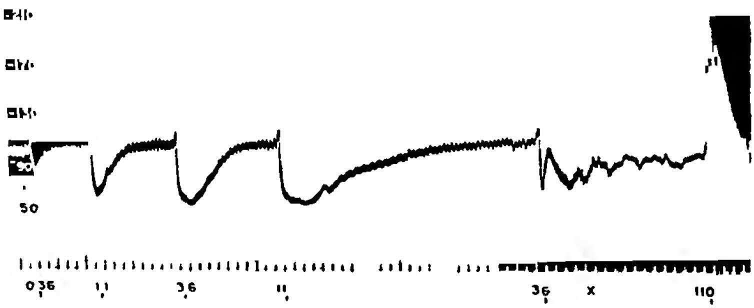

Fig. 1. Effect of Tetraethylammonium Ion on the Arterial Pressure of the Cat (under Dial Anesthetia)

Mercury manometer recording from carotid artery. At the left, scale of pressure in mm. of mercury. Time in minutes. At the signals, intravenous injections of tetraethylammonium bromide in the doses indicated, in mgm. per kgm. At X, generalized muscular fasciculation began.

10 mgm. per kgm. of body weight produce a fall of arterial pressure in cats and dogs. When the dose is larger, the arterial pressure rises (fig. 1). The relation between the dose and the peak depressor or pressor response in cats is shown in figure 2. In this figure the heavy, broken line indicates roughly the mean dose-response relation as determined in 23 experiments. Since only 8 of these experiments included pressor responses, the pressor part of the mean curve is less reliable than the depressor part. The results in dogs under anesthesia with sodium pentobarbital are similar to those illustrated in figures 1 and 2.

Also plotted in figure 2 are the extreme results among the 23 experiments, that is, the results from those animals with the least and the greatest sensitivity to depressor and to pressor doses. The figure therefore indicates not only the nature of the dose-response relation, but also the range of effective doses in various cats. Thus the least effective depressor dose varied from 0 05 to 0.4 mgm.

per kgm., and a dose of 1.0 mgm. per kgm. might produce as little as 10 per cent fall of arterial pressure, or as much as 55 per cent. Furthermore, whereas in 17 experiments 4 mgm. per kgm. or greater doses produced profound depressor responses, one animal had a pressor response to 4 mgm. per kgm. Another animal still had a depressor response to 36 mgm. per kgm.

No correlation was found between the depressor or pressor sensitivities of various animals and either the sex or the mean basal arterial pressure of these

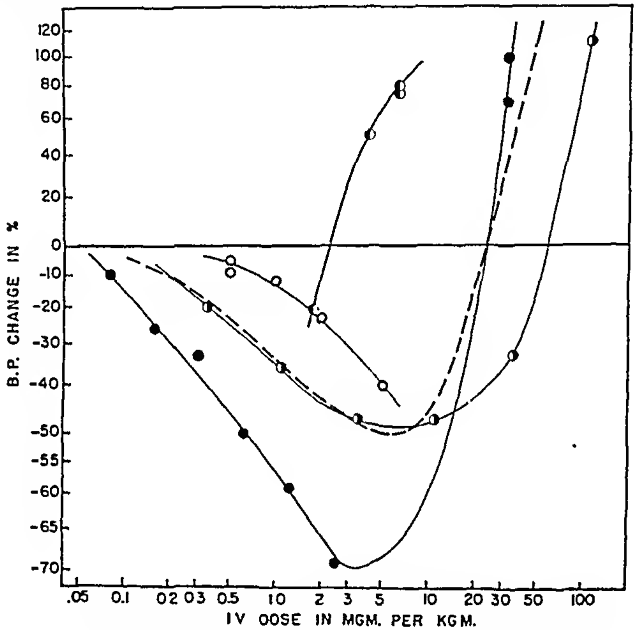

Fig. 2. Dose-Response Relations: Tetraethylammonium Ion on the Mean Blood Pressure of the Cat

Ordinates: change of arterial pressure in per cent, on a logarithmic scale. Abscissae: dose of tetraethylammonium bromide, injected intravenously, in mgm. per kgm., on a logarithmic scale.

The heavy, broken line represents roughly the mean dose-response curve of 23 experiments. The finer lines are plotted from experiments representing the extreme results from this group.

animals. Two animals tested first under ether and again one hour after intercollicular decerebration and removal of the ether (fig. 4A, B), and one tested under chloralose had dose-response relations similar to those of the other 20 animals under dial anesthesia.

The depressor sensitivity and the pressor sensitivity of a given animal bear no constant relation to each other. Thus in figure 2 the animal with greatest depressor sensitivity had an average pressor sensitivity, like that of several other animals which had average depressor sensitivities. And the animal with the least pressor sensitivity had an average depressor sensitivity.

The time characteristics of the depressor responses are functions of the dose. After rapid intravenous injection, the arterial pressure begins to fall within 5 to 15 seconds, reaches its lowest level in ½ to 2 minutes, and returns to the basal level in 2 to 20 minutes. Often the return to basal is followed by a slight elevation of pressure lasting 3 to 10 minutes.

In response to large depressor doses of 3 to 5 mgm. per kgm. the mean fall of pressure among 15 experiments was 53 mm. of mercury (range 28 to 68), that is, 50 per cent of the mean basal arterial pressure of 105 mm. of mercury (range 82 to 137).

When doses of 1 or 2 mgm. per kgm. of tetraethylammonium bromide are injected intravenously in cats every 5 to 10 minutes for an hour, the corresponding depressor responses do not diminish or increase. If, however, a single large dose is injected in the middle of such a series, the subsequent responses may for a time be pressor rather than depressor and do not return to the original form and extent for a long period. Thus when 30 mgm. per kgm. was injected in the middle of a series of injections of 2 mgm. per kgm. every 11 minutes, the responses to the succeeding 6 injections of the smaller dose were pressor. In another experiment, the response to a small dose had not returned to the basal state two and one half hours after an injection of 60 mgm. per kgm.

Continuous intravenous infusion of tetraethylammonium bromide at rates from about 0.1 to 0.5 mgm. per kgm. per min. in cats produces a fall of arterial pressure which begins within the first minute or two and after two to four minutes reaches a steady level which is proportional to the rate of infusion. Recovery upon cessation of the infusion occurs at a rate comparable to that found after single intravenous injections. When the rate of intravenous infusion is greater than 0.5 mgm. per kgm. per min., the arterial pressure falls as described and then rises gradually. With very high rates of infusion of the substance, the pressure may rise above the basal level within a few minutes.

Intramuscular and intraperitoneal injections of tetraethylammonium bromide were performed in 6 cats under dial anesthesia. In 2 of the 6 animals, initial doses of 10 mgm. per kgm. produced moderate falls of arterial pressure lasting 50 and 60 minutes. In another animal an initial dose of 4.5 mgm. per kgm. produced no effect on the arterial pressure; and in the remaining 3 animals the response to doses of 4 to 10 mgm. per kgm. was a rise of 5 to 15 mm. of mercury. The responses to subsequent intramuscular or intraperitoneal injections in the same animals were also variable. Of 12 such observations, depressor responses of 7 to 51 per cent to doses of 10 to 100 mgm. per kgm. occurred in 6; no response occurred in 3 instances with doses of 5, 30, and 30 mgm. per kgm. respectively; and pressor responses occurred in 3 after 30, 50, and 100 mgm. per kgm. respectively.

The usual response of heart rate in anesthetized dogs and cats to intravenous injection of depressor doses of tetraethylammonium bromide is a moderate bradycardia similar in course and duration to the depressor response. In cats and dogs the smallest depressor doses produce no change of heart rate. Bradycardia is seen only with doses greater than about 0.25 mgm. per kgm. Except

for this difference of sensitivity, the dose-response curve for bradycardia is similar to that illustrated in figure 2 for the depressor response. The greatest decrease of heart rate observed was 40 per cent. On three occasions in cats, the response of the heart rate was a moderate tachycardia instead of the usual bradycardia; yet the depressor response was typical. In 2 of these the basal heart rate was unusually low (about 100 per min.) for the cat under dial anesthesia.

Discussion. The results presented so far make it clear that tetraethylammonium ion produces a significant fall of arterial pressure in cats and dogs over a wide range of doses. The response increases in intensity and duration with the dose between 0.1 and 10.0 mgm. per kgm. injected intravenously. Doses of 3 to 5 mgm. per kgm. produce a 50% fall of pressure lasting 20 minutes. Continuous intravenous infusion of the substance at suitable rates causes a fall of arterial pressure to a steady level. The results of intramuscular and intraperitoneal injection are less predictable. The vasodepressor response is usually accompanied by a moderate bradycardia; but occasionally when the initial heart rate is low, tachycardia occurs.

Marshall (13) observed vasodepressor responses similar to those described above, upon the intravenous injection of tetraethylammonium chloride in the rabbit. He reported that the cat is less sensitive to this effect than the rabbit. P. Trendelenburg (17) noted a fall of arterial pressure in the rabbit. Otherwise this effect of tetraethylammonium ion has passed unnoticed.

The bulk of this paper is devoted to an analysis of the site of action of tetraethylammonium ion in producing the vasodepressor response. The possibility that the fall of arterial pressure might result from a negative inotropic effect upon the heart has been excluded in a previous study of the action of tetraethylammonium ion on the heart-lung preparation of the dog (1). Rather than a negative inotropic effect, this substance has a positive inotropic effect when the heart is failing. A minimal positive inotropic effect is produced by a single dose which, if distributed through the blood of the heart-lung preparation, would make a concentration of 1:100,000 (48 micromols per liter). In intact cats and dogs the vasodepressor effect is produced by 0.1 to 10.0 mgm. per kgm., in other words, by an overall concentration of 1:10 million to 1:100,000 (0.48 to 48 micromols per liter). These crude calculations suggest that the largest vasodepressor doses might improve the failing heart in the intact animal.

II. The rôle of the bradycardia. The changes of heart rate which result from the intravenous injection of tetraethylammonium ion may also be excluded as possible causes of the vasodepressor effect of this substance. The bradycardia which usually accompanies the fall of arterial pressure is never marked. The occasional animals in which the response was tachycardia instead of bradycardia emphasize the minor role of heart rate in the production of the fall of arterial pressure.

In the cat and dog, section of the vagus nerves in the neck does not alter significantly either the depressor response or the bradycardia produced by intravenous injections of tetraethylammonium bromide. Removal of the carotid sinus area in the vagotomized cat in two experiments slightly increased the de-

pressor response and the bradycardia. Complete decentralization of the cats heart by vagotomy and excision of the stellate ganglion and the next three pairs of thoracic ganglia abolishes the response of heart rate to small or moderate depressor doses (fig. 6) without diminishing the depressor effect.

The possibility that the vasodepressor and cardiodecelerator responses to tetraethylammonium ion might depend upon the stimulation of receptors in the heart and lungs and in the carotid sinus region is ruled out by these experiments. Thus the mechanism of action of tetraethylammonium ion is unlike that of veratrum alkaloids, which produce a similar cardiovascular response by an action on these receptors (see 9).

III. The peripheral resistance. Since the vasodepressor action cannot be attributed to an effect of tetraethylammonium ion on the heart, this action must be due to a decrease of peripheral circulatory resistance. In order to test the changes in peripheral resistance resulting from the injection of tetraethylammonium ion, carotid arterial pressure and femoral blood flow were recorded in 7 anesthetized dogs weighing 15 to 21 kgm. The basal femoral blood flow was from 45 to 120 cc. per minute.

When tetraethylammonium bromide is injected intravenously in small or large depressor doses, the fall of arterial pressure is usually accompanied by an increase of blood flow in the femoral artery (B and C in figure 3). In one exceptional experiment the fall of arterial pressure was accompanied by a decreased femoral blood flow; in this animal the basal arterial pressure was very low.

Intra-arterial injections in a volume of 0.1 cc. were made into the stream of femoral arterial blood via the distal side-tube of the flow cannula. Control injections of saline produced no changes in systemic pressure or femoral blood flow. Intra-arterial injections of tetraethylammonium bromide in doses of 0.3 mgm. or less per kgm. of body weight did not affect the systemic arterial pressure. In three of the experiments, doses of about 0.5 mgm. per kgm. produced, after a delay of 20 to 40 seconds, a fall of arterial pressure accompanied by an increased flow of blood through the femoral artery. By contrast, the liminal depressor dose by intravenous injection in the series of 7 animals lay between 0.1 and 0.3 mgm. per kgm.

When the arterial pressure did not change as a result of intra-arterial injections of tetraethylammonium bromide, the blood flow in the femoral artery either decreased slightly or did not change (A in figure 3). With larger intra-arterial doses, a similar decrease of flow preceded the delayed depressor response. In one exceptional instance, an injection of 0.5 mgm. per kgm. into the femoral artery produced an increase in femoral blood flow which preceded by a few seconds the systemic depressor response.

The experimental results described above illuminate considerably the mechanism of the fall of arterial pressure produced by tetraethylammonium ion. The flow of blood through the femoral artery is determined by the arterial pressure and by the resistance of the vascular bed into which the blood is being delivered. When the arterial pressure is reduced by agencies which do not affect the resistance of the femoral bed, the flow in the femoral artery decreases. When tetraethyl-

ammonium ion was injected in such a way as to produce a fall of arterial pressure, however, the femoral flow usually increased. This increase of flow in the presence of decreased pressure indicates a marked active vasodilatation in the femoral bed as a result of the systemic injection of tetraethylammonium ion. Since the heart has been ruled out as a cause of the vasodepressor response, we may conclude that the chief cause of this response is vasodilatation.

Intra-arterial injection into the femoral bed provides a means of observing the local effects of a drug on the smooth muscle of the vessels. In most instances the

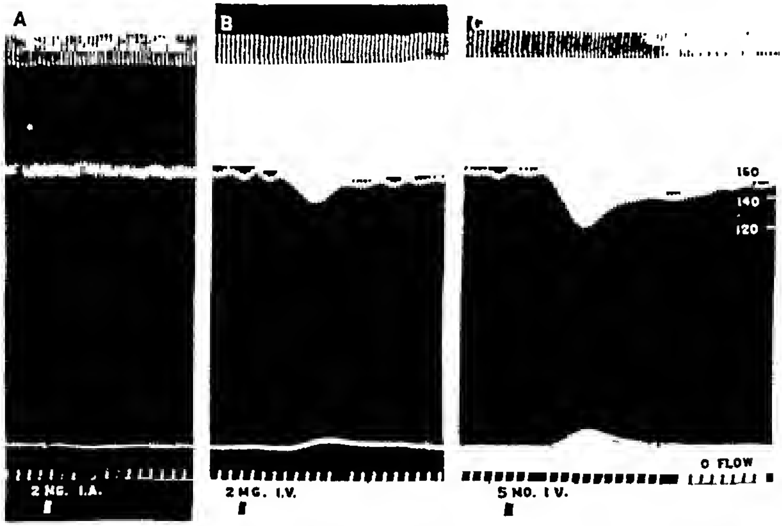

FIG. 3. EFFECTS OF INTRAVENOUS AND INTRA-ARTERIAL INJECTIONS OF TETRAETHYLAMMONIUM ION ON THE SYSTEMIC ARTERIAL PRESSURE AND ON THE FLOW OF BLOOD IN THE FEMORAL ARTERY IN THE DOG (SODIUM PENTOBARBITAL ANESTHESIA)

Top line: respiration as recorded by a lever attached to the chest. Second line: arterial pressure recorded by a mercury manometer from the carotid artery; at the right, scale of pressures, in mm. of mercury. Third line: flow of blood in the femoral artery; for method of recording, see text under Method; upstroke indicates increase of flow. Fourth line: time in 5 second intervals. Bottom line: signal.

At the signals, injections of tetraethylammonium bromide in the doses indicated, in mgm., either intravenously (i.v.) or into the distal arm of the flow cannula in the femoral artery (i.a.).

intra-arterial injection of tetraethylammonium ion produced no change in the blood flow. Some injections led to a decrease in the flow, while the systemic arterial pressure remained constant; hence, in these instances, tetraethylammonium ion had a vasoconstrictor action. Teschendorf observed a slight vasoconstriction when 10 micrograms or more of tetraethylammonium chloride were injected into the perfused hind-leg preparation of the frog (16; see also 13). In the experiments described above no intra-arterial dose of tetraethylammonium ion which did not cause a fall of systemic arterial pressure produced a vasodilata-

tion in the vascular bed into which it was injected. Thus, the local action of tetraethylammonium ion on the vascular bed is, if anything, a vasoconstriction. Consequently, the vasodepressor responses to this substance cannot be explained by its local action on the vascular smooth muscle. The vasodilatation which results from the systemic injection of tetraethylammonium ion is produced by an action elsewhere in the body.

IV. The vasomotor centers. Since intravenous injections of tetraethylammonium ion cause an active dilatation of the arterial bed of the leg by an action elsewhere in the body, it seemed likely that the vasomotor nervous system might be involved in the depressor response. It was therefore desirable to test the possibility that the vasodepressor effect results from an action of tetraethylammonium ion on the medullary centers. Tetraethylammonium bromide was applied to the region of the vasomotor center either by direct application to the floor of the fourth ventricle or by injection into the circulation of the head via the carotid or vertebral artery. The substance was applied to the floor of the fourth ventricle using cats under dial anesthesia in 4 experiments. Doses from 5 micrograms to 5 milligrams in a volume of about 0.02 cc did not significantly affect the arterial pressure. In one experiment an initial dose of 0 2 mgm. led to a slow, moderate rise of arterial pressure, and in another experiment an initial dose of 40 micrograms was followed by a slight fall of pressure and then a slow, slight rise. In each of these experiments subsequent doses did not affect the arterial pressure. After as much as 1 mgm. had been placed in the fourth ventricle, intravenous injections of tetraethylammonium bromide had their usual effect. These large doses of the drug placed in the fourth ventricle led to muscular twitching in the face, tongue, and sternomastoid muscle which began after a pause of 5 to 10 minutes.

In 3 experiments in cats the depressor effect of 0.1 to 0.5 mgm. per kgm. of tetraethylammonium bromide was about the same when injected intravenously as when injected into the arterial circulation of the head.

In two etherized cats, depressor doses of tetraethylammonium bromide were injected intravenously before and an hour after decerebration at the intercollicular level and removal of the ether. The depressor responses were unaffected by removal of the forebrain (fig. 4, A and B).

Thus although high concentrations were capable of stimulating the motor nerves arising in this region, as evidenced by the fasciculation of muscles in the face and neck, no direct action of tetraethylammonium ion upon the vasomotor center was apparent The depressor effect of a given dose was the same whether distributed generally about the body by intravenous injection or whether injected into the circulation of the head.

Since no obvious effect of tetraethylammonium ion on the vasomotor centers was found, it was desirable as a next move to determine the effect of destruction of the medulla on the vasodepressor action.

In 9 experiments in cats (6 anesthetized with ether and 3 with dial), the brain, inclusive of the medulla, was destroyed by pithing from the foramen magnum. This procedure leads to a brief rise of arterial pressure, followed by a fall which

may sometimes proceed gradually for two or more hours before a steady, low level is attained (15, 18). In 4 such experiments, doses of tetraethylammonium ion which are depressor for the intact animal were still depressor after the destruction of the medulla when the arterial pressure was slowly falling. In three of these, doses which would be depressor in the intact animal, injected later, when the arterial pressure had fallen to a lower level, produced either no response, or slight pressor responses. This was the result also in the remaining five experiments (fig. 4, C).

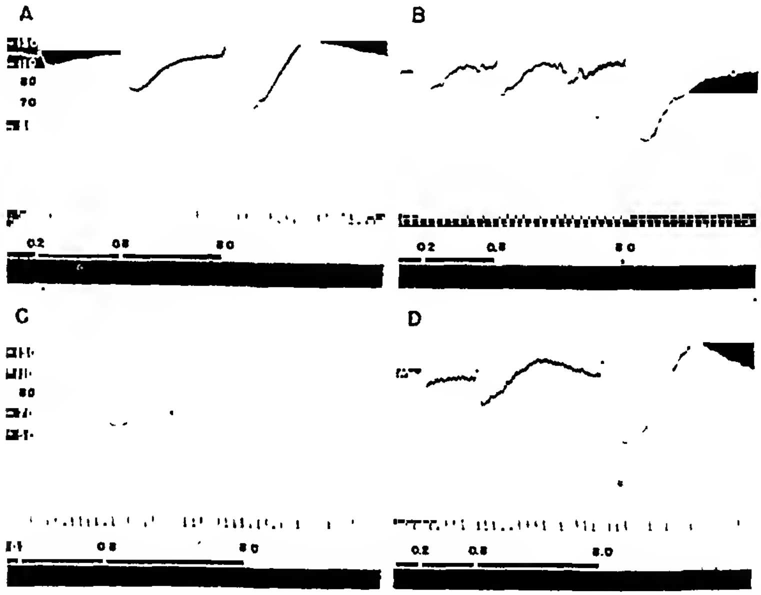

FIG. 4. VASODEPRESSOR EFFECT OF TETRAETHYLAMMONIUM ION ON THE ARTERIAL PRESSURE OF THE DECEREBRATE AND THE SPINAL CAT; RÔLE OF VASOCONSTRICTOR DISCHARGE

Scales at left, arterial pressure in mm. of mercury. Time in minutes. At the signals, intravenous injections of tetraethylammonium bromide in the doses indicated, in mgm.
A. Nervous system intact, light ether anesthesia.
B. One hour after intercollicular decerebration and removal of ether.
C. After destruction of the medulla.
D. During continuous electrical stimulation of cervical spinal cord. Skeletal muscular responses had been eliminated by means of curare.

In two spinal cats, injections of epinephrine bitartrate were made at regular intervals and the pressor responses were recorded. The injection of about 3 mgm. per kgm. of tetraethylammonium bromide shortly before one of these pressor responses did not change the course of this or the subsequent responses of the series. In two other spinal cats, epinephrine bitartrate was injected by continuous infusion so that the arterial pressure was maintained constant near the level of normal pressure. Doses of tetraethylammonium bromide in the depressor range had no effect on the arterial pressure under these circumstances. Doses which are pressor in the intact animal were also pressor here.

Thus when the vasomotor center of the medulla was destroyed, tetraethylammonium ion administered intravenously still produced its vasodepressor response only when a slowly falling arterial pressure gave evidence of a continuing vasoconstrictor discharge from the spinal cord. After the arterial pressure had fallen to a steady, low level, depressor responses could no longer be elicited. Yet the absence of a depressor response did not depend upon the dilated state of the arterioles which obtains when the vasomotor tone is absent; this was demonstrated by the experiments in which the effect of intravenous tetraethylammonium ion was tested during continuous infusion of epinephrine. Despite the normal arterial pressure produced in this manner, the depressor responses were still in abeyance.

The presence of depressor responses in a few animals in which the arterial pressure had not yet reached its basal level after the destruction of the medulla suggests that the mechanism by which the maintenance of arterial pressure is achieved under these circumstances differs in some way from that operating with epinephrine. This difference may consist in a tonic discharge of vasoconstrictor impulses from the spinal cord even after the destruction of the vasomotor center of the medulla. The possibility that the depressor responses to tetraethylammonium ion depend upon the existence of a tonic vasoconstrictor discharge from the spinal cord was tested by injecting doses in the depressor range during electrical stimulation of the cervical cord. In spinal cats, at least one hour after the removal of the ether, wire electrodes were inserted through the opening above the atlas on each side of the upper cervical cord. When strong shocks were applied at frequencies between 1 and 20 per second, the arterial pressure rose rapidly to levels which depended upon the strength and frequency of the stimulation. Simultaneously there was considerable muscular activity. In most of the observations in the 5 experiments of this type, the arterial pressure could not be maintained at a constant level for many minutes; it fell slowly and steadily. The experiment illustrated in figure 4D is unusual in that the arterial pressure remained at a steady level during this stimulation. This experiment is typical, however, in that the injection of tetraethylammonium bromide produced depressor responses similar to those observed in the intact animal. In most of the experiments, on the other hand, the return of arterial pressure to its previous level after the depressor response was incomplete, as might be expected when the basal arterial pressure was slowly falling.

Since the elevation of arterial pressure produced by stimulation of the spinal cord was accompanied by activity of the skeletal musculature, it was important to determine the significance of this activity for the depressor responses to the tetraethylammonium ion. This was accomplished by the use of curare in three of the 5 experiments in which the cord was stimulated. The injection of sufficient crude curare or Intocostrin to prevent the skeletal muscular activity did not affect the depressor responses to tetraethylammonium bromide. In two experiments in intact cats under dial anesthesia, the depressor responses to the substance were likewise unaffected by curarization sufficient to block the activity of skeletal muscles.

Since tetraethylammonium ion is effective in producing a typical vasodepressor

effect in the spinal animal when the arterial pressure is maintained near the normal level by stimulation of the spinal cord but not when the pressure is maintained by epinephrine, it is concluded that a vasoconstrictor tone is an indispensable factor for the demonstration of the depressor response to tetraethylammonium ion. This conclusion makes the role of the vasomotor center clearer. The evidence cited shows a lack of direct effect of tetraethylammonium ion on the vasomotor center; yet when the center is destroyed, the depressor response disappears, when the vasoconstrictor tone disappears. The reestablishment of a vasoconstrictor tone by stimulation of the spinal cord again permits the demonstration of the depressor response. Thus the vasomotor center is not of itself essential for the demonstration of the response; yet it normally supplies a factor which is indispensable, namely, the vasoconstrictor discharge.

V. *Autonomic neuroeffector systems.* The possibility that tetraethylammonium ion might interfere with the vasoconstrictor tone at a peripheral site was studied by testing the effect of this substance on peripheral autonomic neuroeffector systems.

In cats under dial anesthesia, electrodes were placed on the cervical sympathetic nerve. This was crushed lower in the neck and separated from the vagus nerve. Figure 5 illustrates the effect of intravenous injections of tetraethylammonium bromide upon the arterial pressure and simultaneously upon the isotonic contraction of the nictitating membrane produced by stimulation of its preganglionic nerve. The vasodepressor response is paralleled by a relaxation of the nictitating membrane. When the postganglionic fibers are stimulated instead of the preganglionic fibers, the relaxation of the nictitating membrane is absent (see 2).

The effect of tetraethylammonium bromide on the cardioaccelerator and cardiodecelerator nerves was tested in a series of experiments on cats under dial anesthesia. The procedures employed and the results obtained are exemplified in figure 6. In figure 6A, the test dose of tetraethylammonium bromide (1 mgm. per kgm.) produced a moderate bradycardia (A 1). After section of the vagus nerves, the heart rate rose from 193 to 203. Now the test dose produced a more striking bradycardia (A 2). When the preganglionic accelerator fibers were cut (thus completely decentralizing the heart), the heart rate fell to 140. The preganglionic nerves of the stellate ganglion were now stimulated electrically with maximal shocks at a rate sufficient to imitate the previous accelerator tone, i.e., to bring the heart rate up to about 190. The test dose of tetraethylammonium bromide now produced a slightly greater bradycardia (A 3). Finally, when the test dose was injected in the absence of stimulation of the cardiac innervation, the rate of the decentralized heart did not change significantly (A 4).

In figure 6B, the comparison of preganglionic and postganglionic stimulation of accelerator nerves is exemplified, from another experiment. The heart had been completely decentralized. When no stimulation of cardiac innervation was performed, the test dose of tetraethylammonium bromide produced no change of heart rate (B 1). When the preganglionic accelerator fibers were stimulated, the accelerated heart rate which resulted was temporarily diminished by the test dose

(B 2). But when the postganglionic accelerator fibers were stimulated, the test dose of tetraethylammonium bromide was without effect (B 3).

In figure 6 C, the effect of tetraethylammonium ion upon the response to preganglionic stimulation of the right vagus nerve is illustrated from a third experiment. In C 1, the effect of the test dose upon the heart rate of the intact, anesthetized animal is seen to be a slight bradycardia. Section of the vagus nerves

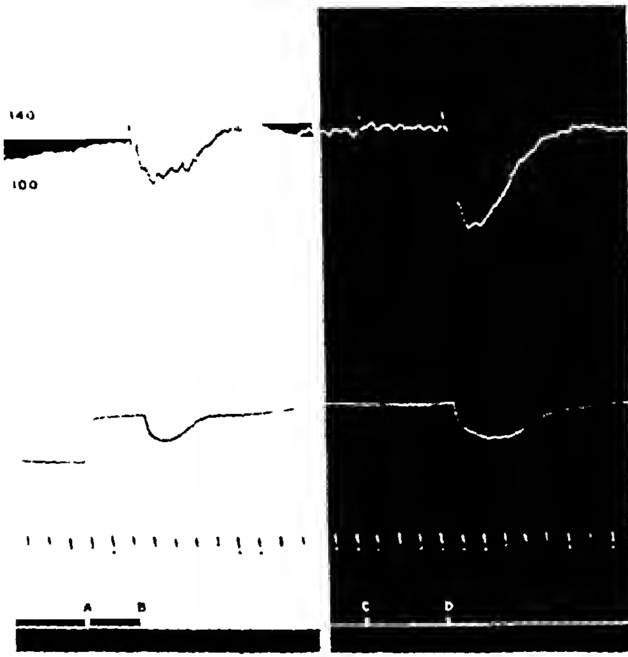

FIG. 5. PARALLEL EFFECTS OF TETRAETHYLAMMONIUM ION ON ARTERIAL PRESSURE AND ON CONTRACTION OF NICTITATING MEMBRANE ELICITED BY STIMULATION OF PREGANGLIONIC NERVE. CAT UNDER DIAL ANESTHESIA

Top line: arterial pressure recorded from mercury manometer; scale at left, pressure in mm. of mercury. Second line: myogram of nictitating membrane. Third line: time in minutes. Fourth line: signal.

At *A*, beginning of stimulation of cervical sympathetic nerve, crushed caudad to stimulating electrodes. At *B*, intravenous injection of tetraethylammonium bromide, 0.3 mgm. per kgm. in 1.5 cc saline At *C*, intravenous injection of 1.5 cc. saline. At *D*, tetraethylammonium bromide 1 mgm. per kgm.

allowed the heart rate to rise from 140 to 184, and now the test dose produced a greater bradycardia (C 2). Section of the accelerator nerves reduced the heart rate again to 140, and now the test dose was without effect on the rate of the (completely decentralized) heart (C 3). Maximal stimulation of the vagus at 1 per sec. produced a fall of heart rate to 113. While the cardio-inhibitory action was present, the test dose of tetraethylammonium bromide produced a rise of heart rate (C 3). In C 4 and C 5, this procedure was repeated during stimulation of

the vagus at 3 and 10 per sec., respectively. The effect of the test dose of tetraethylammonium ion, differed only quantitatively from that illustrated in C 3.

The vasoconstrictor discharge upon which the depressor response to tetraethylammonium ion depends leaves the spinal cord via the sympathetic vasoconstrictor nerves In the sympathetic ganglia the preganglionic sympathetic fibers make synaptic connections with postganglionic neurons. The postgan-

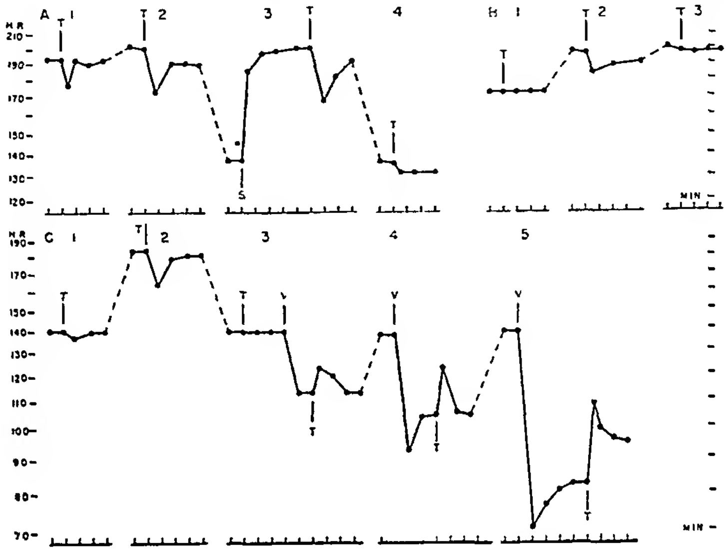

FIG 6 EFFECT OF SECTION AND STIMULATION OF VAGUS AND ACCELERATOR NERVES ON THE RESPONSE OF HEART RATE TO TETRAETHYLAMMONIUM ION CATS UNDER DIAL ANESTHESIA

Ordinates heart rates counted from electrocardiograms Abcissae time in minutes. At T, intravenous injections of tetraethylammonium bromide, 1 mgm per kgm
A Rôle of accelerator tone (1) Cardiac innervation intact (2) After vagotomy. (3) Completely decentralized heart (section of preganglionic fibers to stellate ganglia and removal of thoracic ganglia 2, 3, and 4) during maximal electrical stimulation of preganglionic fibers of R stellate ganglion The rate of stimulation was adjusted to match the heart rate before denervation (4) Same, without stimulation
B Another animal Rôle of ganglionic transmission Completely decentralized heart (1) No stimulation (2) During stimulation of preganglionic accelerator nerves. (3) During stimulation of postganglionic accelerator nerves
C Another animal Rôle of vagal tone (1) Cardiac innervation intact (2) After vagotomy (3) Completely decentralized heart At V, stimulation of vagus nerve at 1 per sec. (4) Same, stimulation of vagus at 3 per sec (5) Same, stimulation of vagus at 10 per sec.

glionic axons carry the impulses to the vascular smooth muscles. In these experiments the preganglionic axons, separated from the central nervous system, were stimulated electrically and the response of the effector was recorded. When a tonic preganglionic discharge was set up in the cervical sympathetic nerve, tetraethylammonium ion produced a fall of tension of the nictitating membrane parallel to the fall of arterial pressure. The same result obtained in the heart

rate when the preganglionic cardioaccelerators were stimulated. Since the response to postganglionic stimulation was unaffected, it may be concluded that in each instance tetraethylammonium ion produced a block of the preganglionic impulses at the sympathetic ganglia. The lack of interference with the effects of impulses in these postganglionic adrenergic nerve fibers recalls the observation reported in section IV that tetraethylammonium ion does not affect the pressor action of epinephrine.

Discussion. *A. The mechanism of the vasodepressor and cardiodecelerator responses.* The analysis of the site of action of tetraethylammonium ion in producing the vasodepressor response has thus led to the conclusion that the substance acts chiefly if not exclusively at the sympathetic ganglia through which the vasoconstrictor impulses from the spinal cord must pass. At these ganglia it sets up a block, which prevents the preganglionic nerve impulses from being transmitted to the postganglionic neurons. This block results in a temporary diminution of the tonic discharge arriving at the arterioles via the postganglionic vasoconstrictor fibers, which keeps the arterial pressure at a normal level. Hence the fall of arterial pressure produced by intravenous injection of tetraethylammonium ion is a consequence of temporary blockage of the vasoconstrictor tone at the sympathetic ganglia.

The data presented in section V indicate that not only the vasoconstrictor system, but also other autonomic neuroeffector systems are subject to this ganglionic block by tetraethylammonium ion. The motor impulses to the nictitating membrane of the cat are interrupted at the superior cervical ganglion; and the accelerator impulses to the heart are interrupted at the stellate ganglion. Furthermore, impulses descending the preganglionic vagus nerve may be blocked in a parallel manner by tetraethylammonium ion; the lack of direct data on vagal postganglionic fibers makes a positive statement on this question unwarranted. Although other autonomic neuroeffector systems have not been tested, it seems justified to predict from the available data that the ganglionic block produced by this substance is widespread in both the sympathetic and parasympathetic nervous systems

Since the vasodepressor responses to tetraethylammonium ion are adequately explained by a ganglionic block in the vasoconstrictor pathway, the changes of heart rate which accompany these responses might well have a parallel explanation. If cardio-accelerator tone predominates, as commonly occurs in cats under dial anesthesia, a ganglionic block should produce cardiac deceleration. This was indeed the usual response of heart rate to tetraethylammonium ion. If on the other hand, cardio-inhibitory discharge predominates, a block of the vagus ganglia should produce cardiac acceleration. In the few instances in which tetraethylammonium ion produced acceleration, the heart rate was unusually low. The results of the experiments involving stimulation of the accelerator or decelerator nerves are adequately explained by ganglionic block. When the rate of the decentralized heart is accelerated by stimulation of preganglionic accelerator nerves, tetraethylammonium ion produces deceleration. And when it is decelerated by stimulation of the preganglionic fibers of the vagus, tetraethylammonium ion produces acceleration. The effects of postganglionic accele-

rator stimulation are unaffected by the substance. The stimulation of postganglionic vagus fibers was not attempted.

B. Side effects of tetraethylammonium ion; relation to other substances affecting the autonomic nervous system. In the course of the experiments on the vasodepressor and cardiodecelerator responses to tetraethylammonium ion, data were collected on its side effects. Since tetraethylammonium ion belongs to the family of simple quaternary ammonium compounds, one might expect it to possess many of the pharmacological properties of its near relatives. One of these, tetramethylammonium ion, resembles nicotine in its actions but also elicits effects reminiscent of muscarine as well as curare. In contrast to this substance, and many other quaternary ammonium compounds, tetraethylammonium ion exerts its characteristic ganglionic block without significant side effects over a wide range of dosage.

Previous reports are in agreement that tetraethylammonium ion lacks entirely the muscarinic action which is characteristic of choline esters and other quaternary ammonium compounds (4, 5, 7, 8). This is confirmed in the present work by the absence of vasodilatation when the substance is injected intra-arterially in the dog's leg (section III), and in this and the previous paper (1) by the absence of a cardiodecelerator effect in the decentralized heart. It is also confirmed by the results of 11 experiments on cats under dial anethesia in which atropine did not have a consistent effect upon the vasodepressor response to intravenous tetraethylammonium ion. In 5 experiments, dose-response relations for tetraethylammonium bromide were examined over a wide dose range before and after a standard large intravenous dose of atropine sulfate (1 mgm. per kgm.). In 3 of these, atropine increased the responses to tetraethylammonium ion; in a fourth, it had no effect on these responses; and in the fifth, it decreased them. In 6 other experiments, single test doses of tetraethylammonium ion were injected before and after the atropine. The vasodepressor response after atropine was increased in one of these, decreased in two, and unchanged in three.

Although an anti-muscarinic, or atropine-like effect, of tetraethylammonium ion has been claimed (11), it has also been denied (4, 5). Its absence was confirmed in experiments in which the effect of intravenous injections of tetraethylammonium ion on the vasodepressor responses to acetylcholine were tested by the method of Kühl (10). In 3 cats under dial anesthesia, small doses of acetylcholine chloride (0.4 to 2 micrograms) were injected intravenously at intervals of 3 or 4 minutes. When a series of equal vasodepressor responses had been obtained as a result of these injections, a dose of 3 to 10 mgm. per kgm. of tetraethylammonium bromide was injected intravenously. The depressor responses to acetylcholine which occurred during the depressor response to tetraethylammonium ion were somewhat smaller than the previous ones. The greater dilatation of the arterioles at the time the acetylcholine was injected accounts for this temporary diminution. As soon as the arterial pressure had returned to its basal level after the response to tetraethylammonium ion, the depressor responses to acetylcholine returned to their original magnitude.

The absence of a "nicotinic-stimulating" action over a wide range of doses of tetraethylammonium ion was apparent from practically every experiment on

which this paper is based. This conclusion confirms the statement of Burn and Dale (3), derived from experiments on the arterial pressure of spinal cats, that with "reasonable doses" the "nicotinic stimulating" action is absent. Pressor responses were produced, however, when the dose was large enough (section I). The minimal pressor dose is somewhere between 40 and 360 times the minimal depressor dose in intact, anesthetized cats (figs. 1 and 2). Enough data are available in cats with the brain pithed, or with both brain and cord pithed, to indicate that, when vasoconstrictor discharge is absent, the minimal pressor dose is smaller than in the intact animal. The mechanism of the pressor response has not been thoroughly analyzed. Yet the vasoconstrictor action, which sometimes appeared when large doses of tetraethylammonium bromide were injected into the circulation of the leg (section III), may play a role in the pressor effect. The important consideration for the present discussion is the absence of a pressor or a "nicotinic-stimulating" action over a wide range of doses of tetraethylammonium ion.

The absence of significant effects upon the respiration places tetraethylammonium ion in sharp contrast to its near relative, tetramethylammonium ion, as was noted by Marshall (13). The latter either stimulates respiration, in a manner suggestive of the "nicotinic-stimulating" action of nicotine and acetylcholine acting at the carotid body, or paralyzes it, like curare. In the experiments upon which this paper is based, muscular paralysis or respiratory failure was never observed with large or small doses of tetraethylammonium ion. When muscular paralysis was produced by curare, the vasodepressor response to tetraethylammonium ion was unaffected (section IV). The question whether tetraethylammonium ion has any true curariform action upon skeletal muscle is discussed by Ing and Wright (6).

Very large doses of tetraethylammonium ion injected intravenously produce muscular fasciculation. A fasciculation of the muscles of the neck and face was observed when the substance was placed in the fourth ventricle in sufficiently high concentration. Fasciculation was reported in the frog by Jacobj and Hagenberg (7), and Loeb and Ewald (12) studied the stimulation of frog nerve by tetraethylammonium ion.

It was noted in section IV that tetraethylammonium ion does not interfere with the pressor response to epinephrine in the spinal cat.

The absence of side effects of depressor doses of tetraethylammonium ion upon the heart has been described in section II. The changes of heart rate are not such as to impair the circulation. No negative inotropic effect is present; and the larger depressor doses are capable of a positive inotropic effect in the failing heart. Only very large doses produce irregularities of the heart (1).

Hunt (4,5) found the subcutaneous lethal dose of tetraethylammonium chloride in mice to be in the neighborhood of 110 mgm. per kgm., far above the doses considered in this paper. Marshall (13) reported that rabbits die when 50 or more mgm. per kgm. of the chloride are injected intravenously over a period of one minute.

Thus tetraethylammonium ion is a non-toxic substance capable of blocking conduction through autonomic ganglia without side-effects over a wide range of

doses. Burn and Dale (3) noted that this substance prevents the nicotinic vasopressor action of tetramethylammonium ion in the spinal cat. Hunt (4) confirmed this "nicotinic-paralyzing" effect of tetraethylammonium ion and showed that other triethylalkylammonium compounds also possess this property. Thus tetraethylammonium ion is capable of blocking the stimulating action upon ganglion cells either of preganglionic nerve impulses or of substances with a "nicotinic-stimulating" action. Since tetraethylammonium ion itself lacks this "nicotinic-stimulating" action, it seems inappropriate to name the predominant action of this substance after a minor action of nicotine. We therefore propose that the predominant action of tetraethylammonium ion, which accounts for its vasodepressor and cardiodecelerator effects, be called simply a ganglionic blocking effect.

SUMMARY

The actions of tetraethylammonium bromide upon the circulatory system of the cat and dog have been described and analyzed. Over a wide range of doses (0.1 to 10 mgm. per kgm.), intravenous injections of this material produce falls of arterial pressure whose magnitude is a function of the dose and which last several minutes. The heart rate is usually diminished moderately, and this effect is absent if the cardiac innervation is severed.

The fall of arterial pressure is shown not to depend upon actions on the heart, the vascular smooth muscle, or the medullary vasomotor center. It results from a block in the ganglia in the efferent pathway of the sympathetic vasoconstrictor nerves. This ganglionic blocking action has been demonstrated in the superior cervical ganglion, the stellate ganglion, and the ganglia of the cardio-inhibitory fibers of the vagus.

The ganglionic blocking action of tetraethylammonium ion is shown to be its most prominent effect. Side effects are absent with all but the largest doses of this substance.

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STUDIES ON THE PHARMACOLOGY OF SALICYLATES[1]

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The initiation of the AAF Rheumatic Fever Control Program brought to light the fact that the existing knowledge concerning the absorption, distribution and excretion of salicylates was insufficient for rational therapy. The salicylates have been used for more than fifty years for rheumatic fever, but it was only recently that Coburn (2) suggested that their use has not been satisfactory, due to the employment of inadequate doses. Opinion is divided regarding the usefulness in rheumatic fever of large doses of salicylates as contrasted with smaller doses (9) (10).

The only extensive study on the metabolism of salicylates is that of Kapp and Coburn (5) (6). They showed that salicylates were excreted partly as free salicylate, partly as salicyluric acid, and partly as glycuronides of salicylic acid. In addition to these forms containing the salicyl radical, two other substances were isolated. Ordinarily, however, normal individuals excrete 80 per cent of the administered salicylate as compounds containing the salicyl radical (6), most of it as salicyluric acid. The recovery of salicyl forms is less in patients with fever, partly due to a diminished excretion of salicyluric acid. They showed, too, that the excretion of salicylates is relatively slow, with about 50 per cent of it exereted in 24 hours, but more than half of this is excreted during the first 12 hours. They found that after some days of sodium salicylate therapy the free fraction increased markedly.

More extensive studies on the absorption, distribution and excretion of salicylates have been handicapped by a lack of simple methods for the determination of these eompounds. Reeently, Brodie, Udenfriend and Coburn (1) published a simple and precise method for the determination of salicylate in plasma. This depends on the extraction of the acidified plasma with ethylene dichloride and development of the color with an iron salt. They showed that after the administration of sodium salicylate the only substance in plasma yielding a color was free salicylate. Using this method, Smull, Wegria and Leland (8) showed that lower serum levels were obtained after the administration of sodium salicylate plus sodium bicarbonate than after the administration of sodium salicylate alone. They suggested that the lower levels were due to one of the following: (a) interference with absorption, (b) increased extracellular fluid volume, or (c) increased renal excretion. Since sodium bicarbonate is employed commonly with sodium salicylate to decrease the gastrointestinal irritation, it seemed important to see if the last mentioned work could be confirmed, and to find out, if possible, what

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effect sodium bicarbonate has. The present study was initiated for this purpose, although it has been extended to other problems that are related to salicylate metabolism.

Chemical methods. The method for determining salicylate in plasma was essentially that described by Brodie, Udenfriend, and Coburn (1). The methods employed for the determination of the salicyl fractions in urine are based on the observations of Kapp and Coburn (5) (6) and those of Brodie, Udenfriend and Coburn (1). Of the salicyl forms in the urine, free salicylate and salicyluric acid give a color with iron salts without hydrolysis whereas the glycuronates do not react until after hydrolysis.

Pure unextracted solutions of salicyluric acid[3] were analyzed and found to give 0.82 as much color as an equal molecular quantity of sodium salicylate. When pure solutions of sodium salicylate were extracted with ethylene dichloride it was found that 0.90 of the salicylate was extracted. This is the same as the figure found by Brodie, et al. (1). The amount of salicyluric acid extracted under these conditions was such that the resulting color was 0.60 of that given by a pure un-

TABLE 1

Hydrolysis of salicyluric acid

HOURS HYDROLYZED	2-LOG G	INCREASE IN 2-LOG G	PER CENT HYDROLYZED
0	0.226	0.000	0
3	0.284	0.058	34
6	0.321	0.095	55
12	0.362	0.136	79
Complete*	0.398	0.172	100

* Molecular equivalent sample of sodium salicylate.

extracted equal molecular quantity of sodium salicylate. Similar extractions were made employing carbon tetrachloride which was known from Brodie's work to extract but a small proportion of the salicyluric acid (1). It was found that the color given with pure sodium salicylate was 0.92 of that given by an unextracted solution, whereas the color given by an equal molecular quantity of salicyluric acid was only 0.04 of that given by sodium salicylate.

It was known that brief hydrolysis with acid would free the salicyl glycuronates (6), but the salicyluric acid is more resistant to hydrolysis. Accordingly experiments were set up to determine the extent of hydrolysis of pure samples of salicyluric acid after varying intervals of time. The results are given in detail in table 1. When the data are plotted on semi-logarithmic paper with the per cent unhydrolyzed as ordinates and time in hours as abscissae a straight line extrapolating to one hundred per cent at zero time fits the data suggesting that the dydrolysis follows the unimolecular law. After three hours hydrolysis and ex-

[3] Samples of salicyluric acid were kindly furnished by Dr. B. B. Brodie, New York University and Lt. Eleanor Kapp Darby, Naval Medical Research Institute, Bethesda, Maryland.

traction with ethylene dichloride the color with salicyluric acid is 0.70 of that given an equal molecular quantity of salicylate which has not been extracted. Since the original salicyluric acid gave 0.60 and the pure sodium salicylate gave 0.90 of the color given by unextracted salicylate, it is obvious that one-third of the salicyluric acid has been hydrolyzed.

Employing the above data it may be seen that readings on a calibration curve, employing ethylene dichloride, of a mixture of sodium salicylate and salicyluric acid should give $SA + \left(\frac{0.60}{0.90} \times 0.82\right) SU$, or $SA + 0.54\ SU$, where SA refers to the free salicylate and SU refers to salicyluric acid. Similarily, readings on the carbon tetrachloride curve give $SA + \left(\frac{0.04}{0.92} \times 0.82\right) SU = SA + 0.04\ SU$. The reading on the carbon tetrachloride curve is subtracted from the reading on the ethylene dichloride curve and the result is $0.50\ SU$. Also readings on the ethylene dichloride curve of hydrolyzed samples will give $SA + 0.33\ SU + SG$, where SG represents the salicyl glycuronates. Summarizing, $SU = 2.00\ (EDC - CCl_4)$; $SA = CCl_4 - 0.04\ SU$; $ST = EDC\ (\text{hydrolyzed}) + 0.33\ SU$, where ST refers to total salicyl fractions as salicylate.

Mixtures of sodium salicylate and salicyluric acid were prepared and the resulting values came within 5 per cent of those predicted from the above equations. In addition known amounts of sodium salicylate and salicyluric acid were added to a sample of urine from a rheumatic fever patient. The results were calculated according to the above equations and they agree with those predicted by the equations within approximately 5 per cent. Hydrolysis of the urine for a time longer than 3 hours gave an increase in readings, but these were of the order to be expected from the amount of salicyluric acid in the samples, suggesting that all of the salicyl glycuronates have been hydrolyzed in the three hour period. Detailed directions for the determination of the salicylate in plasma and of the salicyl fractions in urine follow:

Standard solutions. Sodium salicylate solution; 1.160 gm. of sodium salicylate dissolved in water and made up to one liter. One cc. contains 1 mg. of salicylate. Salicyluric acid solution; 0.141 gm. salicyluric acid made up to 100 cc. One cc. is equivalent to 1 mg. of salicylate.

Procedure for plasma. Place 1 cc. of plasma, 1 cc. of water, and 0.5 cc. of 6 N HCl and 30 cc. of ethylene dichloride in a 60 cc. glass stoppered Pyrex bottle. Shake vigorously for five minutes on a shaking apparatus. Transfer the mixture to a 50 cc. centrifuge tube and centrifuge for five minutes at moderate speed. Remove the supernatant aqueous layer by aspiration. Transfer exactly 20 cc of the ethylene dichloride layer to a dry 60 cc. glass stoppered bottle and add 10 cc. of water and 0.25 cc. of the iron reagent. Shake for five minutes. Transfer at least 6 cc. of the supernatant aqueous layer to a colorimeter tube. Read in the Evelyn colorimeter using a filter with maximal transmission at 540 mu. Read from the EDC curve for plasma.

Procedure for salicyl fractions in urine. a. The urine is diluted 1 to 10 or 1 to 20 and the extraction is carried through with ethylene dichloride as for plasma and the values are read on the EDC curve of the graph.

b. A similar procedure is used with carbon tetrachloride as the solvent, and the results are read on the CCl_4 curve of the graph.

c. A procedure similar to that with ethylene dichloride is employed but, instead of dilute acid and water, 0.5 cc. dilute urine and 1 cc. of concentrated HCl are added and the mixture hydrolyzed for three hours by immersing the lower part of the bottles into the steam bath. After cooling, 1.0 cc. of water and 30 cc. of ethylene dichloride are added, and the procedure is carried out as above. The readings are then made on the standard curve for EDC, and multiplied by the appropriate dilution factor. The results are calculated as below:

$$\begin{aligned} SU &= 2.00\ (EDC - CCl_4) \\ SA &= CCl_4 - 0.04\ SU \\ ST &= EDC\ (\text{hydrolyzed}) + 0.33\ SU \end{aligned}$$

As an incidental finding it was found that chloroform was as useful as ethylene dichloride in the determination of plasma salicylates. This is of some practical importance since chloroform is more readily obtained in many hospital laboratories than ethylene dichloride.

DISTRIBUTION IN TISSUES, BLOOD AND PLASMA. The distribution of salicylates in tissues was studied in rats killed approximately two hours after single oral doses or after the animals had eaten food mixed with sodium salicylate for several days. In the latter case the food was removed about four hours before the animals were killed. The salicylates were determined in the tissues by grinding up the tissue with sand prior to the addition of acid and ethylene dichloride. The chlorides were determined after digestion with a mixture of concentrated nitric acid and silver nitrate with concentrated potassium permanganate added until the mixture was clear.

In order to determine the amount of salicylate in the erythrocytes, blood samples were taken from patients receiving salicylate, using heparin as an anticoagulant. Apparent cell volumes were determined as well as whole blood and plasma salicylates, and from this the amount in the red cells was calculated.

Studies of the binding of salicylate by plasma proteins were carried out using the ultrafiltration method of Lavities (7) employing a cellophane membrane. The method of calculation is as follows:

(1) mg. salicylate in plasma water per L. plasma =

$$\frac{\text{wt. water per cc. plasma}}{\text{wt. water per cc. ultrafilt.}} \times \text{mg. salicylate per L. ultrafilt.}$$

(2) mg. salicylate in residue water per L. residue =

$$\frac{\text{wt. water per cc. residue}}{\text{wt. water per cc. ultrafilt.}} \times \text{mg. salicylate per L. ultrafilt.}$$

(3) mg. salicylate per L. plasma associated with non-diffusible portion = mg. salicylate per L. plasma − (1)

(4) mg. salicylate per L. residue associated with non-diffusible portion = mg. salicylate per L. residue − (2)

(5) mg. salicylate per gm. plasma non-diffusible solids =

$$\frac{(3)}{\text{dry wt. 1 cc. residue} - (\text{dry wt. 1 cc. ultrafilt.} \times \dfrac{\text{wt. water in 1 cc. plasma}}{\text{wt. water in 1 cc. ultrafilt.}}}$$

(6) mg. salicylate per gm. residue non-diffusible solids =

$$\frac{(4)}{\text{dry wt. 1 cc. residue} - (\text{dry wt. 1 cc. ultrafilt.} \times \dfrac{\text{wt. water in 1 cc. residue}}{\text{wt. water in 1 cc. ultrafilt.}}}$$

Distribution in tissues. When sodium salicylate was given to rats, either as a single oral dose, or mixed with food, the concentrations of salicylate in the tissues were, as may be seen from table 2, almost as high in some tissues as would be predicted if the concentration of salicylate in the tissue water was in equilib-

rium with the concentration of salicylate in the serum. This is approximately true for liver, kidney, lung, and whole blood, but the concentrations in muscle and in brain are approximately one-half as high as might be predicted on the basis of the water content.

TABLE 2

Concentration of chloride, salicylate and water in the tissues of rats after oral administration of sodium salicylate

Mean cell volume, 44

	MEQ. Cl PER KG. FRESH TISSUE IN:						
	Serum	Liver	Muscle	Kidney	Brain	Lung	Whole blood
Mean	100	26	14	47	31	52	
	PER CENT WATER IN:						
	Serum	Liver	Muscle	Kidney	Brain	Lung	Whole blood
Mean		71	77	75	79	80	
	MG. SALICYLATE PER KG. FRESH TISSUE IN:						
	Serum	Liver	Muscle	Kidney	Brain	Lung	Whole blood
Single doses	360	220		285		275	305
	2,420	1,389	1,419	1,631		2,002	
	720	595	297	585	253	655	640
	940	689	243	659	362	806	
Drug in food	200	91	18	160	54	90	160
	300	166	86	298	139	205	
	360	225	110	240	121	208	
	270	146	56	188	83	201	260
	SALICYLATE CONCENTRATIONS DIVIDED BY WATER CONTENT:						
	Serum	Liver	Muscle	Kidney	Brain	Lung	Whole blood
Single doses	392	306		380		344	424
	2,630	1,930	1,845	2,178		2,506	
	783	826	386	780	320	820	890
	1,022	957	316	879	458	1,004	
Drug in food	218	126	23	213	68	113	222
	326	231	112	398	176	256	
	393	313	143	320	153	260	
	294	203	73	251	105	253	361

Ultrafiltration studies on human plasma. It may be seen from the results in table 3 and the graph in figure 1 that there is a rapid increase in the ultrafiltrable

salicylate compared with that in the whole plasma as the concentration of salicylate in plasma increases. At plasma levels of 200 mg. per liter less than 50 mg. per liter is in the ultrafiltrate, but at a plasma level of 500 mg. per liter almost one-half as much is found in the ultrafiltrate. When the amount of salicylate per gram of non-diffusible solids (presumable plasma proteins) is calculated it may be seen from figure 2 that the amount of salicylate per gram of plasma proteins rises with the concentration of salicylate in the plasma. There is a slight tendency for the curve to level off, but there is no evidence that, even at levels of 500 mg. per liter, the amount of bound salicylate has reached a maximum.

TABLE 3

Amount of salicylate bound to human blood plasma proteins

MG. SALICYLATE PER LITER			MG. SALICYLATE PER GM. NON-DIFFUS. SOLIDS	
Plasma	Ultrafilt.	Residue	Plasma	Residue
166	32	320	1.66	1.60
185	32	324	1.82	1.77
160	32	248	1.46	1.38
105	25	210	0.95	1.12
96	26	220	1.01	1.30
150	30	250	1.39	1.57
175	45	340	1.50	1.64
252	84	400	2.02	2.00
380	110	654	3.02	2.98
312	94	510	2.84	2.86
418	148	650	3.15	3.11
434	156	686	3.65	3.57
436	166	654	3.80	3.42
526	165	906	4.22	4.14
502	242	680	3.54	3.21
480*	216	654	3.58	3.52
512†	214	780	3.75	3.68

* In vitro experiment in which salicylic acid and ammonium chloride were added to a sample of salicylate-free plasma. The final pH was 7.41.

† In vitro experiment as above but sodium salicylate was added and the final pH was 8.12.

The point arose as to whether the combination of salicylate with plasma proteins was the result of somd metabolic effect. Also no attempts were made to prepare the ultrafiltrates on freshly drawn plasma. Accordingly salicylic acid and ammonium chloride were added to a sample of salicylate free plasma and the final pH as determined on the glass electrode was 7.41. To another, sodium salicylate was added and the final pH was 8.12. Ultrafiltrates were prepared from these samples and the results are given as the black circles in figures 1 and 2. It may be seen that, within the limits of the experimental errors, the ratio of plasma salicylate to ultrafiltrate salicylate and the amount of salicylate bound per gram of non-diffusible solids is the same as in the plasma of patients receiving salicylate. This suggests that whatever the forces are, binding the salicylate to

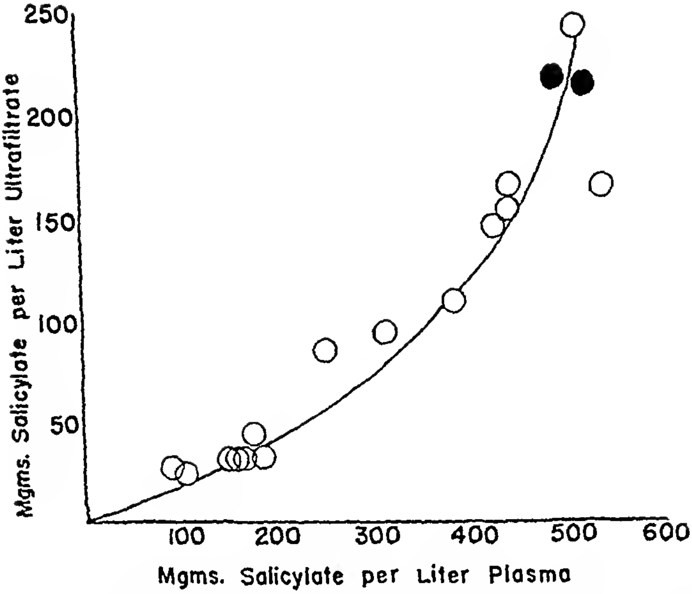

FIG. 1. RELATIONSHIP BETWEEN SALICYLATE IN PLASMA AND PLASMA ULTRAFILTRATES
Black circles represent in vitro experiments

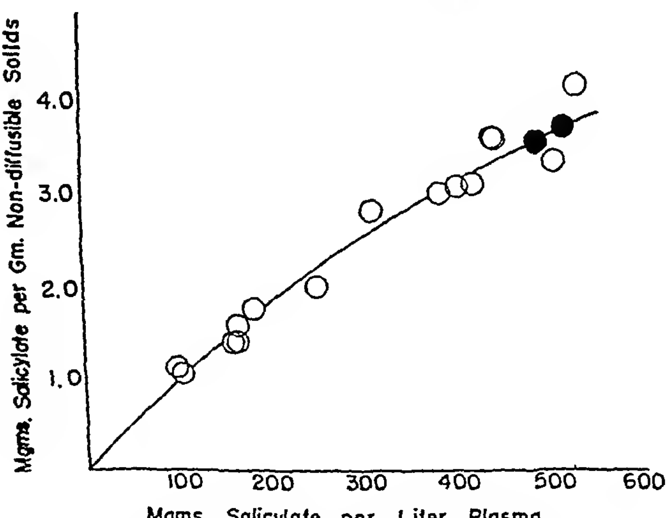

FIG. 2. RELATIONSHIP BETWEEN SALICYLATE CONCENTRATIONS IN PLASMA AND AMOUNT OF SALICYLATE BOUND TO PLASMA PROTEINS
Black circles represent in vitro experiments

the plasma proteins, the formation of complexes does not depend upon bodily processes and is not very sensitive to variations in pH in the range studied.

Concentrations in human erythrocytes. As may be seen from table 4, the amounts in the red cells were quite low, and are of the same order of magnitude as might be expected if the cell membrane were freely permeable to the salicylate contained in the plasma ultrafiltrate. This, of course, is not sufficient evidence that such a condition exists.

Absorption and excretion. *Single doses.* Sodium salicylate or aspirin in 2 gram doses was given to aviation students on an average of two hours after breakfast. The results of these experiments with sodium salicylate are summarized in figure 3. As is already generally known, the absorption of sodium salicylate is rapid; appreciable concentrations being found in the plasma in thirty minutes. Peak levels are reached in approximately two hours, and thereafter the levels fall slowly over the rest of the eight hour period studied. The variation in plasma concentrations was considerable. No account was taken of variations in body weight. The rapid absorption after oral administration is an effective argument against the intravenous administration of the drug except in patients who are unable to retain it after oral administration. The comparatively slow decline in plasma level suggests that the administration of the drug every six hours will be often enough to maintain reasonably constant plasma levels.

TABLE 4

Concentrations of salicylate in human erythrocytes

PLASMA	MG. SALICYLATE PER LITER CELLS	CELLS*
125	20	18
127	12	17
166	3	28
157	23	26
191	25	34
148	12	23
180	22	31
162	10	27

$$\text{Concentration in cells} = \frac{\text{whole blood conc.} - (\text{plasma vol.} \times \text{plasma conc.})}{\text{cell volume}}.$$

* Calculated on the assumption that the salicylate in erythrocytes is merely that dissolved in the cell water and is in equilibrium with the salicylate in the plasma ultrafiltrate.

In contrast to the results with sodium salicylate, the data on aspirin (fig. 4) indicate that when salicylate is given in this form it is more slowly absorbed. The slower rise of the plasma salicylate curve after aspirin than after sodium salicylate suggests that the acetyl-salicylate may be hydrolyzed first before absorption, and that, after approximately four hours, excretion becomes as rapid as absorption so that during the remaining four hours the plasma levels do not fall appreciably. The maximum plasma levels obtained with aspirin are appreciably less than those obtained with the same doses of sodium salicylate, but it is

to be remembered that there are two factors that would tend to make this so. First, the amount of salicylate in 2 grams of aspirin is appreciably less than the amount in 2 grams of sodium salicylate, and secondly, the slower absorption of

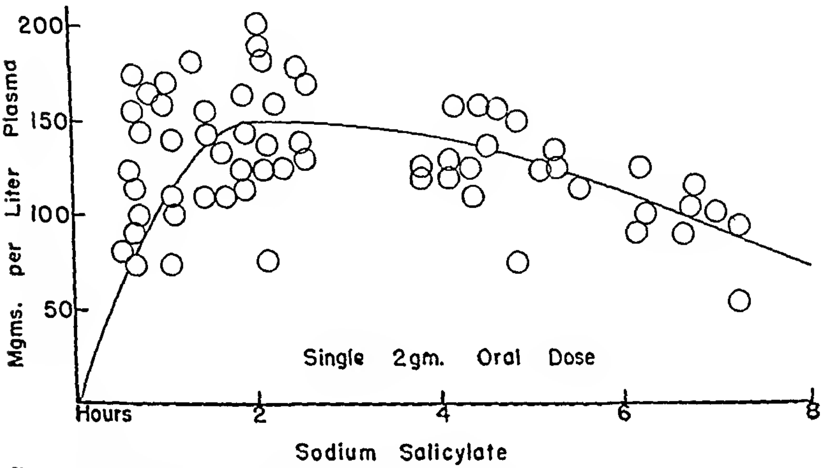

FIG. 3. PLASMA CONCENTRATIONS AFTER SINGLE 2 GM. DOSES OF SODIUM SALICYLATE TO NORMAL ADULTS

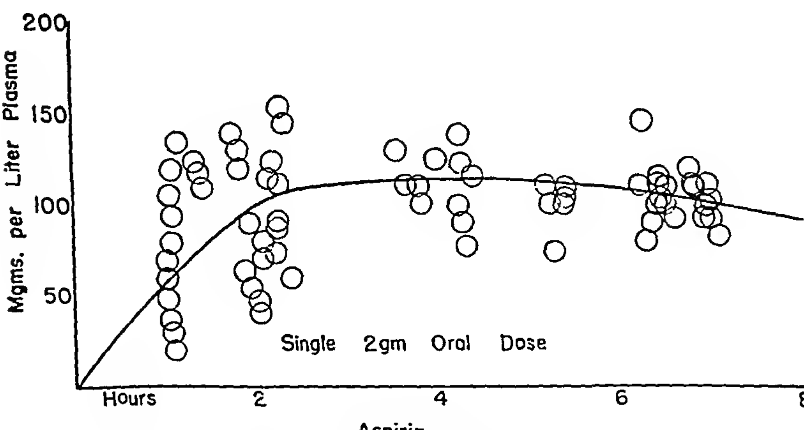

FIG. 4. PLASMA CONCENTRATIONS OF FREE SALICYLATE AFTER SINGLE 2 GM. DOSES OF ASPIRIN

aspirin permits the greater excretion of the compound during the time it is being absorbed.

The data on aspirin are particularly interesting because it is often stated that aspirin is absorbed to an appreciable extent in unchanged form, whereas the

above experiment suggests that in the plasma it is primarily in the form of salicylate. Further experiments were carried out to elucidate this point. First, it was determined that when aspirin was added directly to serum and extracted with ethylene dichloride very little color was obtained when iron was added. Secondly, it was found that when aspirin was added to serum and the mixture was hydrolyzed with hydrochloric acid, as in the procedure for total salicylate in urine, most of the aspirin was converted to free salicylate (recovery of 460 mg. per liter when 500 mg. per liter was added). With this informaion it was possible to utilize the method to gain further information. Salicylates in the plasma of patients receiving aspirin were determined before and after hydrolysis and the results in twelve cases gave no evidence that aspirin as such exists in the plasma (mean of 101.4 mg. per liter before hydrolysis and 99.1 mg. per liter after hydrolysis and with an average difference of 3.8 mg. per liter).

The above experiments did not eliminate the possibility that the aspirin was absorbed as such and then hydrolyzed in the tissues. Attempts to gain further

TABLE 5

Plasma concentrations of salicylates and urinary concentrations of salicyl fractions after the oral or intravenous administration of aspirin in dogs

DOG	MODE OF ADMINISTRATION	PLASMA SALICYLATE		URINARY FRACTIONS			PER CENT OF READILY HYDROLYZABLE SALICYLATE	pH
		Before hydrolysis	After hydrolysis	Free Salicylate	Salicyluric acid	Total Salicylate		
		mg. per liter		*mg. per liter*				
A	Oral	360	350	495	126	818	24.1	6.2
A	Intrav.	452	445	630	525	2,000	42.2	6.1
B	Oral	160	155	495	125	913	32.1	6.7
B	Intrav.	315	320	304	62	620	41.0	6.7
C	Oral	255	260	558	138	696	1.4	6.8
C	Intrav.	220	220	1,035	75	2,050	45.9	6.8

information on this point were made using dogs. Three dogs were each given aspirin neutralized with sodium bicarbonate immediately before oral administration. One to two hours later blood samples were taken on the animals and plasma salicylate determinations done before and after hydrolysis. Salicyl fractions were determined in the urine. Analogous experiments were done on the same dogs except that the aspirin and sodium bicarbonate were given intravenously. As may be seen from table 5 the data on the plasma levels suggests that the aspirin is hydrolyzed in the tissues to salicylate since neither after oral or intravenous administration was any measurable concentration of bound salicylate found in the serum. Approximately the same relative concentrations of the salicyl fractions are found in the urine after oral as after intravenous administration, suggesting that the metabolism of the drug is the same whatever the mode of administration. It may be noted, however, that after intravenous injection the per cent of readily hydrolyzable salicylate (presumably glycuronides and aspirin) is in all cases greater, suggesting that some aspirin is excreted unchanged. The

above information suggests that the actions of aspirin as an analgesic, and in the treatment of rheumatic fever, may be no different than those of other simple

TABLE 6

Plasma concentrations of salicylate and urinary excretion of free salicylate, salicylurate and total salicyl compounds

DRUG	NO. OF CASES	WT.	MG. SALICYLATE PER LITER PLASMA				
			1	2	3	4	5
		kgm.					
Sodium salicylate	9	69.1	104	171	240	225	123
Sodium salicylate plus sodium bicarbonate	9	69.8	107	189	228	151	61
Sodium salicylate plus ammonium chloride	6	77.8	113	185	244	268	191
Ammonium salicylate	6		107	189	247	248	157
Acetylsalicylic acid	10	74.4	107	178	248	238	137

FIRST SAMPLE					SECOND SAMPLE				
cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST
		gm.	gm.	gm.			gm.	gm.	gm
740	6.9	0.49	0.33	1.11	690	6.7	0.57	0.32	1.36
790	7.1	0.51	0.49	1.21	567	7.0	0.57	0.56	1.56
1,063	6.3	0.47	0.23	0.91	892	6.0	0.63	1.15	1.18
670	5.8	0.68	0.12	1.00	620	5.7	0.89	0.16	1.48
456	6.1	0.44	0.12	0.72	670	5.4	0.79	0.08	1.24

THIRD SAMPLE					FOURTH SAMPLE				
cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST
		gm.	gm	gm.			gm.	gm.	gm.
783	6.9	0.72	0.58	1.86	464	6.1	0.54	0.21	1.09
692	7.3	0.50	1.12	2.13	295	6.6	0.39	0.16	0.87
1,014	5.7	0.58	0.10	1.16	488	5.5	0.60	0.05	1.17
531	5.6	0.81	0.22	1.58	367	5.3	0.78	0.02	1.20
573	5.8	0.68	0.22	1.33	525	5.3	0.74	0.05	1.19

TOTAL ALL SAMPLES			
cc.	SU	SA	ST
	gm	gm.	gm.
2,677	2.33	1.46	5.33
2,344	1.89	2.28	5.58
3,457	2.28	0.51	4.42
2,188	3.18	0.50	5.26
2,224	2.54	0.47	4.46

salicylates, such as sodium salicylate. It does not preclude the fact that aspirin may be absorbed as such and then hydrolyzed in the tissues.

In retrospect it is interesting to find that the probable source of the idea that aspirin is absorbed and excreted unchanged arises from some early experiments of Hanzlik and Presho (4). Subjects were given aspirin and the amount of the salicylates in the urine as determined by the iron reaction was measured before and after hydrolysis. The amount after hydrolysis was appreciably greater than the amount before hydrolysis. It is now evident from the work of Kapp and Coburn (6) that what they were actually determining was the glycuronate fractions.

Multiple doses. In order to determine the effects of the simultaneous administration of alkaline and acid salts on the metabolism of salicylates, convalescent

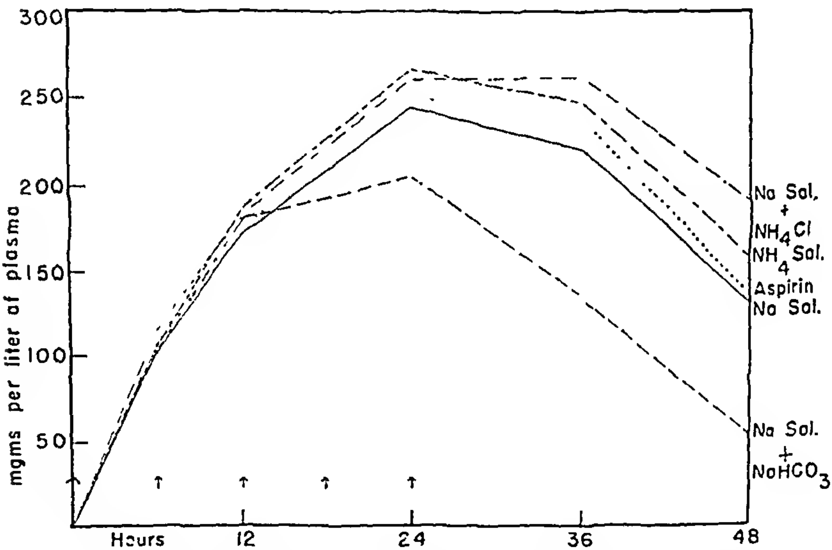

FIG. 5. PLASMA CONCENTRATIONS OF SALICYLATE AFTER THE ADMINISTRATION OF 5 DOSES OF VARIOUS SALICYLATES AT 6-HOUR INTERVALS (INDICATED BY ARROWS)

patients without fever[4] were each given one of various salicylates in five doses of 2 grams each, six hours apart. The plasma levels and urinary concentrations of salicyl fractions were determined during the administration of the drug and for the following twenty-four hours. The results are summarized in table 6 and in figure 5. From the figure showing the plasma levels it may be seen that there was no appreciable difference in the levels obtained during the first twelve hours but twenty-four hours after administration ceased, the plasma levels of the patients receiving sodium salicylate plus sodium bicarbonate were less than half

[4] *These were convalescent patients in the medical service of Lt. Col. M. O. Starr of the San Antonio Aviation Cadet Center to whom the salicylates were administered under the supervision of Major Stoll.*

of those in patients receiving either sodium salicylate alone, aspirin, ammonium salicylate, or sodium salicylate plus ammonium chloride. From an inspection of the results on the amount of the salicyl fractions excreted several differences may be observed. First, the amount of free salicylate excreted when sodium bicarbonate is given along with sodium salicylate is appreciably greater than when the salicylate is given in any other form. The next greatest amount is after sodium salicylate alone. Secondly, the amount of salicyluric acid excreted is greatest after the administration of ammonium salicylate and is least when sodium salicylate is given with sodium bicarbonate. Some of the reasons for these differences will be discussed later in the study on rheumatic fever patients.

If it is assumed that the salicyl glycuronate fraction is equal to the total salicylate minus the sum of free salicylate and salicyluric acid then it may be seen that the average total salicyl glycuronates excreted after the various salicylates are remarkably similar, varying only from 1.41 grams after sodium salicylate

TABLE 7

Plasma concentrations of salicylate and urinary excretion of free salicylate, salicylurate and total salicyl in patients being treated for rheumatic fever

WT.	DOSES, GM. PER 6 HRS.	MG. PER L. PLASMA			FIRST URINE SAMPLE				
		1	2	3	cc.	pH	SU	SA	ST
kgm.							gm.	gm.	gm.
90.9	3.0	330	250	375	605	7.0	0.63	0.56	1.42
77.2	3.0	440	290	440	425	6.9	0.34	0.96	1.42
72.8	2.5	440	290	470	490	6.0	0.75	1.20	1.47
79.1	2.5	510	400	535	210	6.9	0.20	0.64	1.11
78.2	2.5	410	295	380	385	6.8	0.29	0.55	1.06
71.8	2.0	445	300	450	625	6.8	0.30	0.94	1.43
68.2	3.0	530	265	225	835	6.4	0.91	1.22	3.35
76.3	2.5	465	210	235	1,160	6.5	0.83	1.45	2.79
69.1	2.5	360	135	120	920	6.5	0.96	0.61	2.16
66.8	2.5	515	245	225	925	6.2	1.06	0.79	2.99

SECOND URINE SAMPLE					THIRD URINE SAMPLE					FOURTH URINE SAMPLE				
cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST
		gm.	gm.	gm.			gm	gm.	gm.			gm.	gm.	gm.
175	5.8	0.33	0.05	0.59	395	6.2	0.81	0.40	1.64	375	8.0	0.43	1.65	2.32
130	5.8	0.28	0.08	0.61	360	6.2	0.55	0.45	1.58	1,025	7.8	0.88	4.58	5.60
300	5.8	0.63	0.08	1.29	215	6.4	0.33	0.35	1.04	475	8.2	0.36	2.65	3.15
160	7.1	0.17	0.55	0.88	255	6.2	0.18	0.48	1.10	395	6.9	0.23	1.77	2.71
150	6.0	0.19	0.01	0.59	695	6.2	0.79	0.46	1.97	440	7.5	0.15	1.04	1.32
250	6.0	0 36	0.27	0.89	115	5.8	0.25	0.07	0.59	860	7.8	1.63	5.10	7.12
230	6 4	0.23	0.26	0.76	340	5.8	0.58	0.37	2.02	375	6.9	0.53	1.59	2.78
310	5.8	0.35	0.19	0.91	380	5.8	0.54	0.27	1.30	475	7.2	0.36	1.46	1.86
270	5.8	0.77	0.16	1.45	265	5.8	0.77	0.23	1.56	865	7.2	0.82	1.70	3.05
					595	5.9	0.96	0.47	2.42	690	6.7	0.72	1.35	2.49

TABLE 7—*Concluded*

FIFTH URINE SAMPLE					SIXTH URINE SAMPLE					SEVENTH URINE SAMPLE				
cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST
		gm.	gm.	gm.			gm.	gm	gm.			gm.	gm.	gm.
600	7.6	0.91	2.42	3.74	325	6.4	0.57	0.29	1.21	425	5.3	0.55	0.07	0.94
310	7.1	0.41	1.32	1.92	300	5.9	0.44	0.40	1.27	300	6.6	0.37	0.17	0.84
900	7.6	1.03	4.19	5.79	375	6.0	0.66	0.37	1.52	310	6.2	0.49	0.06	0.92
360	7.5	1.03	2.84	4.26	325	6.5	0.49	1.28	2.42	200	6.8	0.28	0.07	0.80
500	7.2	0.31	0.94	1.34	410	6.2	0.41	0.23	0.90	510	5.6	0.39	0.06	0.72
175	7.0	0.25	0.71	1.19	210	6.3	0.24	0.43	1.03	200	5.6	0.21	0.05	0.61
690	7.4	0.72	2.87	5.45	150	7.3	0.31	0.69	1.22	230	6.8	0.63	0.69	1.98
470	7.0	0.36	0.74	1.18	396	6.8	0.49	0.65	1.37	1,105	7.2	0.74	3.12	4.41
760	7.2	0.90	1.86	3.57	140	6.5	0.51	0.22	1.04	445	7.1	1.02	1.12	2.07
630	7.0	0.60	1.61	2.32	415	7.2	0.55	1.22	1.95	520	6.8	1.04	1.23	2.85

EIGHTH URINE SAMPLE					NINTH URINE SAMPLE					TOTAL GIVEN	GM. SALICYLATE RECOVERED			PER CENT RECOVERED
cc.	pH	SU	SA	ST	cc.	pH	SU	SA	ST		SU	SA	ST	
		gm.	gm.	gm.			gm	gm.	gm.		gm.	gm.	gm.	
600	5.9	0.51	0.13	1.05	525	5.6	0.77	0.28	1.69	23.3	3.19	7.91	15.10	65
800	8.1	0.27	0.53	1.04	250	8.7	0.14	0.29	0.54	23.3	5.60	8.44	17.93	77
700	8.3	0.57	0.43	1.07	500	6.2	0.78	0.19	1.68	19.4	3.68	8.78	14.85	76
400	6.4	0.34	0.15	0.98	255	6.4	0.27	0.13	0.84	19.4	3.98	8.45	15.04	78
675	7.4	0.29	0.26	0.73	475	6.4	0.43	0.47	1.19	19.4	5.41	5.85	14.60	75
820	8.3	0.43	0.56	1.27	435	5.6	0.31	0.27	0.90	15.5	3.25	3.92	9.82	63
670	7.8	0.83	2.88	4.06	505	7.6	0.62	1.52	2.35	23.3	5.36	13.09	23.97	103
895	8.1	0.60	2.17	2.50	530	6.2	0.35	0.97	1.48	19.4	7.09	10.42	20.84	108
770	7.9	0.95	2.58	3.66	750	7.9	0.82	1.41	3.91	19.4	4.62	11.02	17.80	92
775	7.8	1.43	2.36	3.66	590	7.8	0.73	1.39	2.16	19.4	7.52	9.89	22.47	116

plus sodium bicarbonate to 1.63 grams after sodium salicylate plus ammonium chloride. The total amount of salicylate excreted under these conditions is greatest after sodium salicylate plus sodium bicarbonate (5.58 gms.), but this amount is not appreciably greater than the amounts after sodium salicylate alone (5.33 gms.) or after ammonium salicylate (5.26 gms.).

After the administration of 10 grams of various forms of salicylates, the average amount recovered up to twenty-four hours after administration of the last dose, is approximately 5 grams of salicyl fractions, out of a total of 7.6 to 8.6 of salicyl administered. Recoveries would undoubtedly have been better if collection of urine had continued for a longer period of time, since in all cases, appreciable concentrations of salicylate were still in the plasma. From the data presented after the administration of single 2 gram doses of sodium salicylate or aspirin, it seems reasonable to suppose that the remaining plasma levels are of the order of those given by the administration of 2 grams of salicylate. With these assumptions, it seems likely that approximately 80 per cent of the salicylates can be recovered in the urine in the form of salicyl compounds.

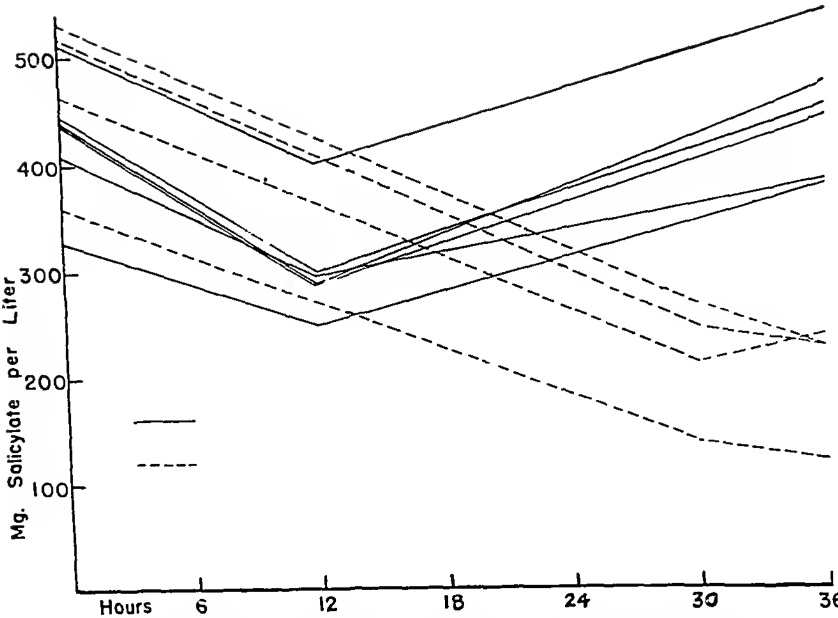

FIG. 6. PLASMA CONCENTRATIONS OF SALICYLATE IN RHEUMATIC FEVER PATIENTS RECEIVING SODIUM SALICYLATE AT 6-HOUR INTERVALS

Patients represented by solid lines received sodium bicarbonate for first 12 hours and patients represented by dashed lines received sodium bicarbonate for first 30 hours.

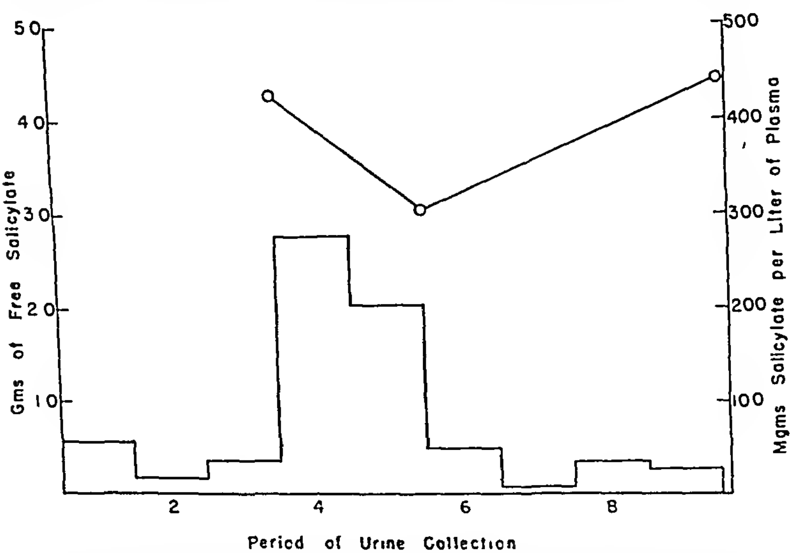

FIG. 7. THE AMOUNT OF FREE SALICYLATE EXCRETED IN PATIENTS RECEIVING THE SAME DOSE OF SODIUM SALICYLATE EVERY 6 HOURS BUT WHO RECEIVED IN ADDITION SODIUM BICARBONATE DURING THE 4TH AND 5TH PERIOD AND AMMONIUM CHLORIDE DURING THE 6TH AND 7TH PERIODS

Rheumatic fever patients. In order to investigate further the effects of the administration of acids and alkalis on the metabolism of salicylates, experiments were carried out on rheumatic fever patients at Truax Field.[5] None had fever during the time these experiments were being done. The patients were given sodium salicylate in the same dose that they had been receiving for some time, and urine was collected over six hour periods, the end of each collection period

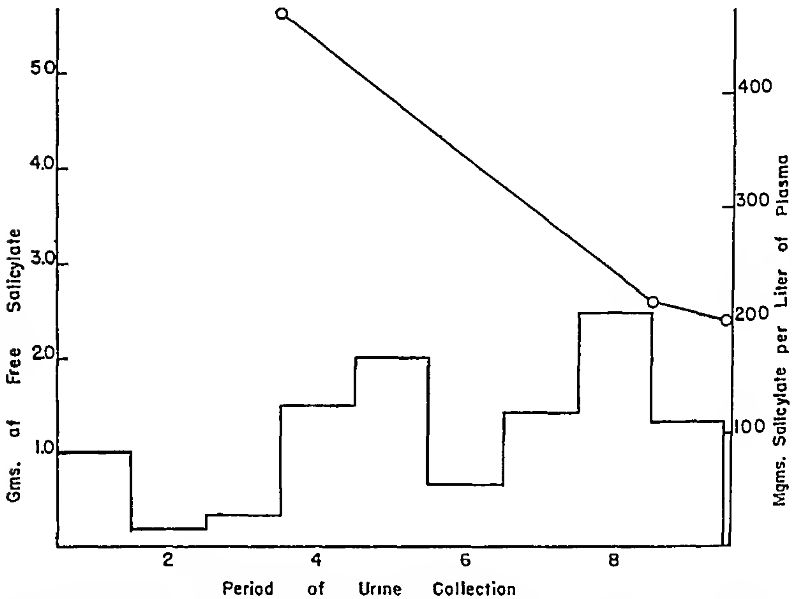

FIG. 8. THE AMOUNT OF FREE SALICYLATE EXCRETED IN PATIENTS RECEIVING THE SAME DOSE OF SODIUM SALICYLATE EVERY 6 HOURS BUT WHO RECEIVED IN ADDITION SODIUM BICARBONATE DURING THE 4TH, 5TH, 6TH, 7TH, AND 8TH PERIODS

coinciding with the administration of the drug. Urine collections were made for three six hour intervals while the patients received sodium salicylate alone. At the end of this time, blood samples were taken on all patients and the administration of sodium bicarbonate, 3 grams every three hours, was begun in addition to the sodium salicylate. To six patients the administration of bicarbonate was continued for twelve hours after which the administration of ammonium chloride, 3 grams every three hours, was begun. To the remaining four sodium bicarbonate was given every three hours for thirty hours after which it was discontinued, and sodium salicylate alone was given. The results are summarized

[5] These were in the medical service of Major H. A. Warren. The laboratory facilities utilized were under the supervision of Major F. S. Coombs.

in table 7. The individual results of the plasma are illustrated in figure 6. In those who received sodium bicarbonate for twelve hours only, there was an appreciable fall in the plasma salicylate level and after the sodium bicarbonate was discontinued and the ammonium chloride begun, there was a rise in the

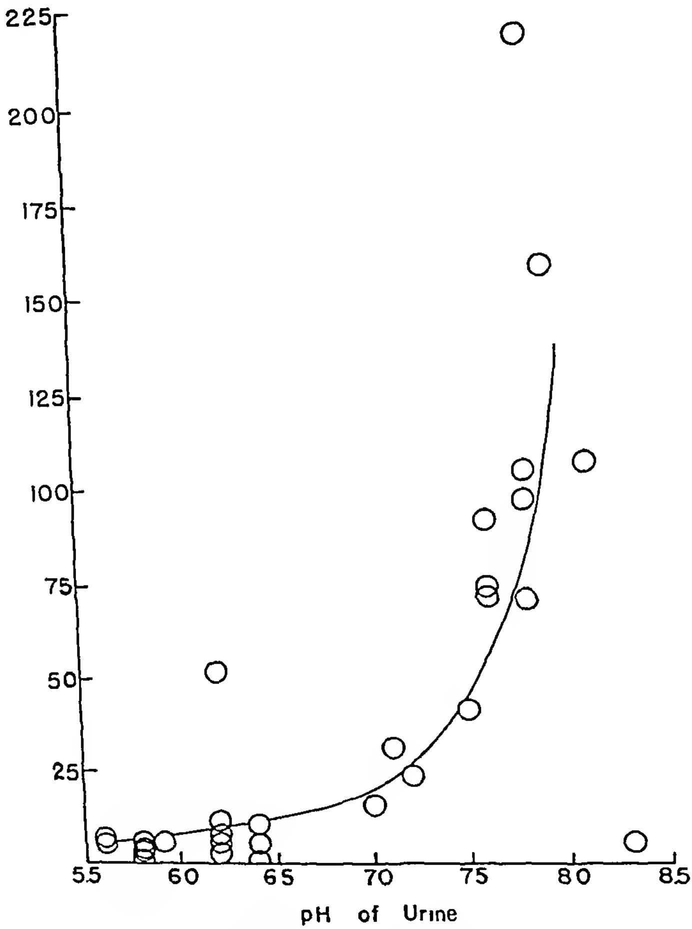

FIG 9 RELATIONSHIP BETWEEN URINARY pH AND RENAL CLEARANCES OF FREE SALICYLATE

plasma level, and the final levels twenty-four hours after sodium bicarbonate administration ceased, were as high as at the beginning of the experiment. The plasma salicylate levels of those who received sodium bicarbonate for thirty hours fell continually during the entire time, and thirty hours after administra-

tion of sodium bicarbonate began, the salicylate levels in the plasma were less than one half the initial values. With the discontinuance of the administration of sodium bicarbonate the decrease in plasma levels during the next six hours was less than it had been during the previous time. This shows clearly that the plasma levels decrease when sodium bicarbonate is given. Inspection of figure 7 shows why this is true, at least in part. As soon as administration of sodium bicarbonate began at the end of period three, there is a large increase in the amount of free salicylate in the urine. The average increase is approximately two grams of salicylate in six hours. Quantitatively, this is approximately enough to account for the decrease in plasma levels. Similar, but not as striking results are observed in the four patients who received sodium bicarbonate for a longer period of time (fig. 8). The average increase in excretion of free salicylate is quite appreciable (1.1 gm.) and is approximately of the order necessary to account for the decrease in plasma levels of salicylates. The administration of sodium bicarbonate was not associated with an increase in the salicyluric acid fraction or the salicyl glycuronate fraction. The amount of total salicyl fractions recovered during the time of the experiment is shown in table 7. In the patients receiving sodium bicarbonate most of the time the apparent recovery is sometimes more than 100 per cent.

Attempts were made to analyze in terms of known kidney functions the excretion of free salicylate. As a preliminary step the milligram of free salicylate per minute excreted was calculated and plotted against the pH of the urine. It was obvious from these data that there was a marked dependence of excretion on the pH of the urine with a gradual rise from pH of 6 up to 8. A more precise analysis, in terms of renal clearances, was introduced by estimating from the curve in figure 1 the concentrations of salicylate in the plasma water and calculating clearances on this basis. The results are shown in figure 9. Again it may be seen there is a marked dependence of excretion rate upon urinary pH.

SUMMARY

1. Colorimetric methods have been developed for the determination in urine of free salicylate, salicyluric acid and total salicylates.

2. Most of the salicylate in human plasma is bound to the non-diffusible components, presumably plasma proteins.

3. Even at high plasma salicylate concentrations very little salicylate is in human erythrocytes.

4. After the oral administration of aspirin to human subjects or oral or intravenous administration to dogs the only detectable form of salicylate in the plasma is free salicylate.

5. The administration of sodium bicarbonate along with salicylates results in lower plasma levels than when the salicylate alone is given.

6. The administration of sodium bicarbonate to patients receiving sodium salicylate results in an increased excretion of free salicylate, with the renal clearances of free salicylate increasing rapidly above pH 7 in the urine.

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PHARMACODYNAMIC STUDIES OF A NEW ANTIHISTAMINE AGENT, N'-PYRIDYL-N'BENZYL-N-DIMETHYLETHYLENE DIAMINE HCl, PYRIBENZAMINE HCl

I. Effects on Salivation, Nictitating Membrane, Lachrymation, Pupil and Blood Pressure

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The suggestion of Dragstedt and others (1) regarding the probable release of histamine in certain allergic conditions encountered clinically and the conclusion that the many manifestations of such allergies were due to the histamine released, accelerated the efforts of varied investigators in their search for an adequate antagonist of this noxious imidazole. Prominent among the results of these efforts in positions of varied therapeutic vantage are histaminase (2), hapamine (3) and antergan (4). Several other promising agents are available which are capable of nullifying to some extent the actions of histamine under experimental conditions. These have been studied by Staub and Bovet (5), Lehman and Young (6), Loew and coworkers (7). R. L. Mayer, C. P. Huttrer and C. R. Scholz (8) have investigated many pyridyl compounds, the most potent of which was N'-pyridyl-N'-benzyl-N-dimethylethylene diamine HCl, called Pyribenzamine HCl. A number of experimental studies (9) have revealed its antihistaminic and anti-anaphylactic properties and the present paper is concerned with further pharmacologic studies of this substance.

Method. Adult cats were anesthetized with sodium pentobarbital, urethane or Dial with urethane. The last two were generally found to be most desirable, especially as concerned secretory responses, and were employed throughout most of the experiments. The animals were prepared surgically as previously described (10) for studying several ipsilateral functions controlled by the same cervical sympathetic nerve, namely, salivation, retraction of the nictitating membrane, lachrymation, and mydriasis. Blood pressure was simultaneously recorded with the Anderson (11) glass membrane manometer. Most injections of the various drugs were made into the ipsilateral carotid artery but occasionally, drugs were injected intravenously and these have been so specified in the text. The drugs, injected in varying concentrations in terms of micrograms per kilogram of body weight, included histamine phosphate, the hydrochlorides of Pyribenzamine, epinephrine, acetylcholine and pilocarpine, and the sulphate of atropine. Faradization of either the cervical sympathetic or the chorda tympani nerves for five or ten seconds was effected by the Harvard inductorium. Mydriasis was observed and salivation, which was measured manometrically (1 cm.—0.0345 cc.), was transcribed upon the kymograph with blood pressure and the responses of the nictitating membrane. These reactions appear in the accompanying figures from above downward in the following order: nictitating membrane, salivation and blood pressure. Time has been recorded in one minute intervals.

RESULTS. From 35 experiments, the following observations have been made:

(1) Pyribenzamine, 10 to 200 micrograms, had no effect on the normal functions studied, except for minor fluctuations in blood pressure after the smaller doses, or a sustained rise following the larger doses (fig. 1).

(2) Histamine, in the optimal dose of 10 micrograms, usually promoted salivation. Occasionally an animal was encountered in which a markedly hypotensive dose of histamine was absolutely without effect as a sialogogue. This occurred most frequently in the most deeply anesthetized animals. Often in this situation, however, other drugs and faradization were still effective.

In general, control responses with histamine were successively quite uniform at 15, 20 or 30 minute intervals but at times, a marked diminution or absence of salivation resulted unpredictably (fig. 2); it seemed of no special significance since invariably these lessened responses or failures of response to histamine were successively sustained following an adequate dose of Pyribenzamine (fig. 3). Best results with histamine were obtained when each injection was preceded by one or two "priming" stimulants in the form of faradization of the chorda tympani nerve for 5 or 10 seconds.

(3) Pyribenzamine, 5 to 20 micrograms, usually nullified the salivary stimulation induced by equivalent or smaller amounts of histamine and this suppression of salivation usually prevailed for about 30 to 120 minutes or more, approximately in proportion to the dose (figs. 3 and 4).

(4) Atropine, 200 micrograms subcutaneously, also prevented the stimulation of salivation by histamine, confirming previous findings of Gibbs and McClanahan (11). No tolerable amount of histamine could break through this atropine block.

(5) Faradization of the chorda tympani nerve and injections of pilocarpine during the suppression of histamine-induced salivation by Pyribenzamine still resulted in salivation (fig 5).

(6) In several experiments Pyribenzamine sensitized the secretory response to pilocarpine, 1 to 2 micrograms, to epinephrine, 15 micrograms, and to faradization of the cervical sympathetic and chorda tympani nerves. This feature prevailed during Pyribenzamine's suppression of histamine-induced salivation (fig. 5).

(7) Histamine, 10 to 50 micrograms or more, frequently, but by no means consistently, retracted the nictitating membrane and this effect was usually nullified or dampened by Pyribenzamine in equal or greater amounts (fig. 6).

(8) In two of our experiments, Pyribenzamine, in a 10 microgram dose, seemed to sensitize the retractile response of the nictitating membrane to epinephrine (fig 7) In this experiment, salivation was simultaneously enhanced.

(9) Uniform mydriasis induced by histamine and observed in approximately 50% of our experiments, prevailed unchanged after Pyribenzamine, 10–50 micrograms

(10) Pyribenzamine, 10 to 50 micrograms, definitely suppressed the lachrymation induced by histamine This suppression seemed to endure as long as histamine-induced salivation was inhibited.

(11) As a rule, Pyribenzamine, 10 to 50 micrograms, had no consistent protective effect against the transient hypotension which invariably followed either the intracarotid or intravenous administration of 10 micrograms of histamine. Figure 4, however, shows such protection to a marked degree; furthermore, 200 micrograms of Pyribenzamine significantly elevated blood pressure but offered

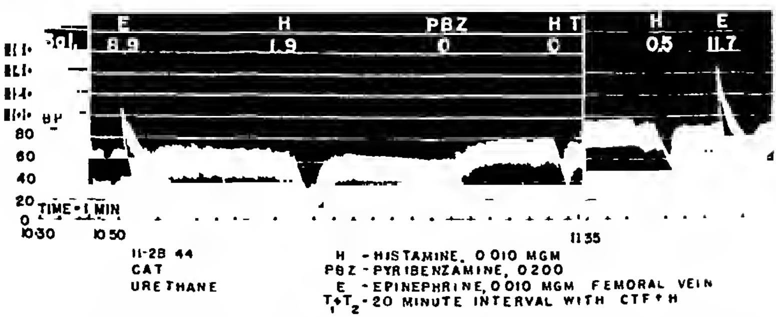

Fig. 1. 11/28/44. Cat, 3.2 kg., urethane anesthesia. From above downward: salivation in centimeters, arterial pressure, time in minutes. Salivation induced by histamine, 10 micrograms, was nullified by Pyribenzamine, 0.200 mgm. and simultaneously salivation as induced by epinephrine, 0.010 mgm., was enhanced by Pyribenzamine as was the attending hypertensive action of epinephrine. At *T*, a time interval of 20 minutes had elapsed during which chorda tympanic faradization and histamine, 10 micrograms, were exercised.

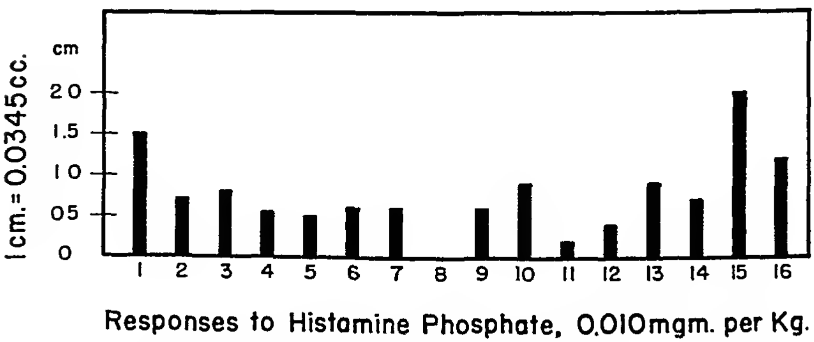

Fig. 2. Histamine Salivation in Cat

12/5/44. Cat, 2.82 kg., urethane anesthesia. The effects of intracarotid administration of histamine phosphate, 10 micrograms per kilogram, on salivation. Note the extremes, ranging from zero to 2.0 centimeters. One centimeter represented 0.0345 milliliter of saliva.

only slight protection against the acute histamine-hypotension (fig. 1). Thus, the vascular effects of Pyribenzamine were variable and independent of dosage.

Discussion. Since only the salivary action and not the effect of nervous stimulation or vagomimetic drugs is antagonized by Pyribenzamine, this drug cannot be designated as atropinmimetic.

This antagonism between histamine and Pyribenzamine probably represents a simple competition-phenomenon of the type recently proposed by Wells, et al., (12) which is similar to that accepted for atropine-acetylcholine antagonism.

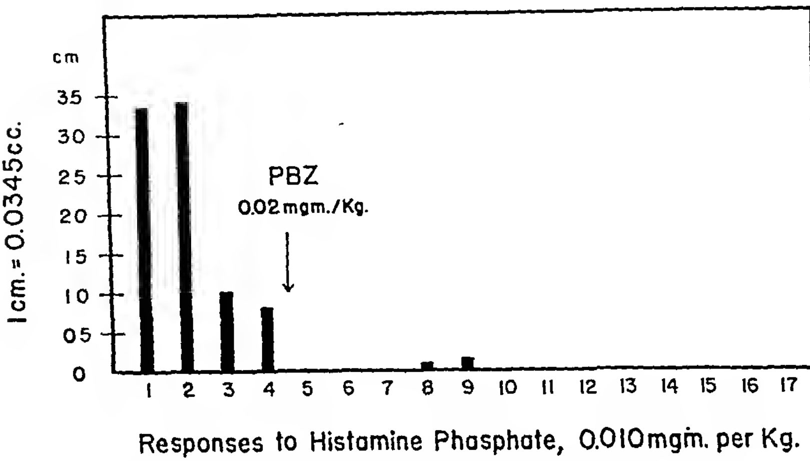

FIG. 3. EFFECT OF PBZ ON HISTAMINE SALIVATION IN CAT

12/27/44. Cat, 3.4 kg., urethane anesthesia. The effects of intracarotid administration of 20 micrograms per kilogram of Pyribenzamine on histamine-induced salivation. Note the sustained suppression of salivation by Pyribenzamine; recovery had not occurred with even the thirteenth injection of histamine following Pyribenzamine.

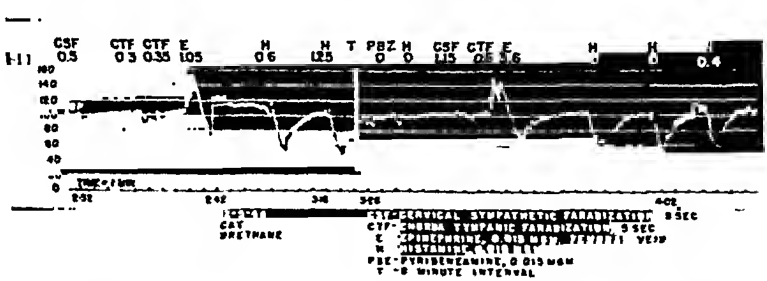

FIG. 4. 12/5/44. Cat, 2.82 kg, urethane anesthesia. From above downward: salivation in centimeters, arterial pressure, time in minutes. Salivation induced by histamine, 10 micrograms was inhibited by Pyribenzamine, 15 micrograms, and marked but temporary protection was afforded against histamine-provoked hypotension. After Pyribenzamine, salivation induced by epinephrine and by faradization of the chorda tympani and cervical sympathetic nerves was enhanced. At T, a time interval of 8 minutes had elapsed.

It is difficult to account for some of the failures of salivary response to successive control doses of histamine. In some of these experiments successive control responses were gradually or acutely diminished, or occasionally were entirely absent. Hence, control injections (fig. 2) were made until it seemed assured that once histamine had promoted salivation, it could be depended upon to do

so quite consistently in 5 out of 6 or in all six successive injections; this was obviously mandatory if proper interpretation were to be made of consistent, successive failures of histamine to induce salivation following Pyribenzamine.

Not infrequently salivary responses were very slight, on the order of 0.05 to 0.1 cm. These values, which represented 0.0017 and 0.0035 cc. respectively, may have reflected true secretory efforts on the part of the gland but in the light of the contribution of Babkin and McKay (13), these minor increments of mano-

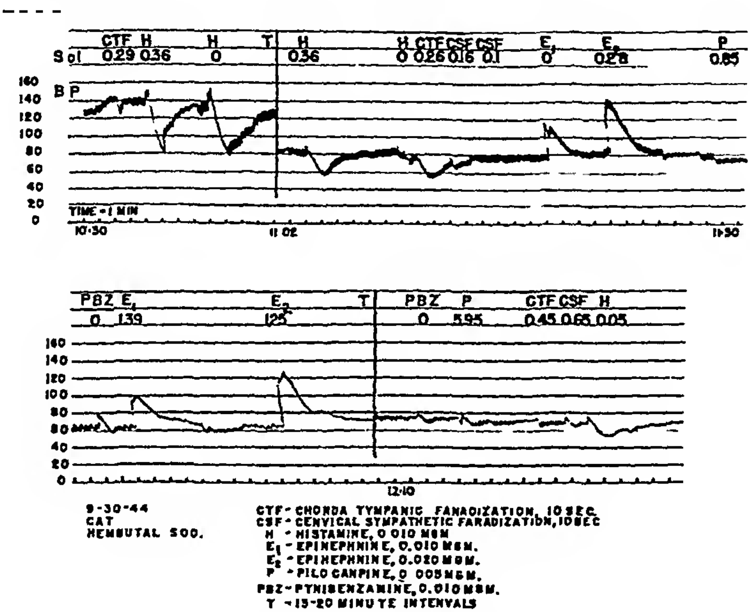

FIG 5 9/30/44 Cat, 2 35 kg, sodium pentobarbital anesthesia. From above downward salivation in centimeters, arterial pressure, time in minutes Faradization of the chorda tympani and cervical sympathetic nerves was not only effective during the paralytic action of Pyribenzamine, 0 010 mgm, on salivation induced by histamine 0 010 mgm, but salivary responses to such faradization were enhanced Responses to epinephrine, 0 010 mgm, and pilocarpine, 0 005 mgm, were likewise markedly enhanced in the presence of antihistaminic doses of Pyribenzamine At T, time intervals of approximately 15 to 20 minutes had elapsed during which faradization of the above nerves and histamine were repeatedly exercised

metric levels might have been merely "expression secretions" resulting from contraction of muscular components in the gland and ducts under stimulation of various types Nevertheless, they have been included here as salivary responses

The antisalivary effect of Pyribenzamine was immediate, as a rule, and usually endured longer than suppression of the histamine-induced retraction of the nictitating membrane, indicating variability in the end-organ's responsiveness to this antihistaminic agent

In the presence of salivary suppression induced by Pyribenzamine, the gland

still responded to pilocarpine and epinephrine as it did to faradization of the cervical sympathetic and chorda tympani nerves. These responses would indicate that Pyribenzamine is specifically antihistaminic in its pharmacodynamic action and apparently did not directly depress salivary gland tissue or act anti-parasympathomimetically like atropine.

Accompanying this salivary suppressive action of Pyribenzamine another pharmacodynamic potentiality of this compound manifested itself in several of our experiments, namely, an apparent sensitizing of the salivary response to pilocarpine, acetylcholine, epinephrine and to sympathetic and parasympathetic

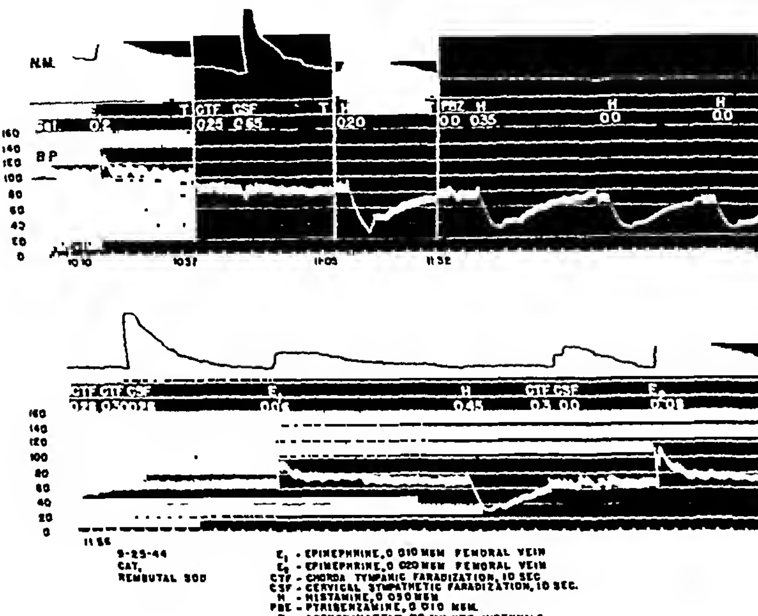

FIG 6 9/25/44 Cat, 2 1 kg, sodium pentobarbital anesthesia From above downward nictitating membrane, salivation, arterial pressure, time in minutes Pyribenzamine, 0 010 mgm, inhibited retraction of the nictitating membrane produced by histamine, 0 050 mgm T, intervals of approximately 20 minutes

faradization In two of our experiments we also observed significant potentiation of the retractile response of the nictitating membrane to epinephrine, but no such changes were observed in respect to the pupil and lachrymation. This potentiation suggests that if the same conditions were to present itself in the human subject, caution should be exercised in the allergic patient, particularly should he be inclined toward the already predominantly adrenergic type in any one of the sympathetically controlled functions. This might be particularly true of the patient suffering from neurogenic hypertension. On the other hand, should such sensitization prevail clinically in the non-hypertensive, allergic pa-

tient, an obvious advantage might be obtained from the sparing or potentiation of normally produced epinephrine or sympathin, whose fortified sympathetic actions might thus duplicate artifically administered epinephrine in the bronchial asthmatic or urticarial subject.

Just how some adrenergic end-organs are primed by Pyribenzamine in their exaggerated responses to their normal sympathetic stimulants is unknown but it seems plausible that Pyribenzamine may well duplicate some of the functions of

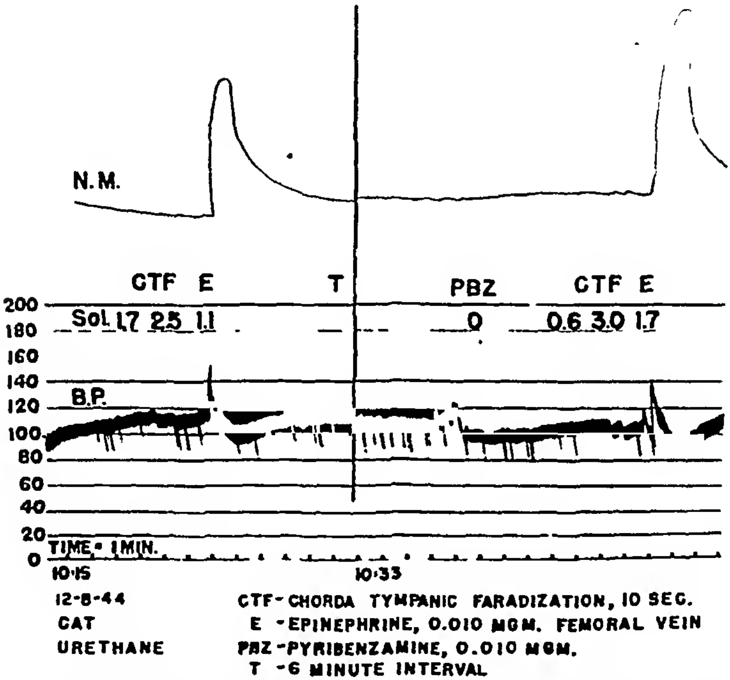

FIG. 7 12/8/44 Cat, 2 67 kg, urethane anesthesia From above downward: nictitating membrane, salivation, arterial pressure, time in minutes Salivation after epinephrine, 0.010 mgm, was slightly increased after Pyribenzamine 0 010 mgm, and retraction of the nictitating membrane was markedly enhanced.

ergotoxine or yohimbine in that, as has been suggested, they may dampen certain enzymic systems associated with amine oxidase, phenolase, tyrosinase or other substances which normally may contribute to the destruction or degradation of epinephrine and sympathin or similar agents. Thus, Pyribenzamine may act in respect to sympathetic stimulation in a manner similar to the action of physostigmine in its relation to parasympathetic stimulation. This seems probable because of the concomitant parasympathetic sensitization observed in some of our experiments. Such increased cholinergic salivation has been observed

before (14) in our experiments dealing with the sympatholytic properties of ergotamine tartrate.

Although the salivary gland, nictitating membrane and lachrymal gland are not necessarily concerned in the problem of allergy, they lend themselves readily to an experimental study of histamine antagonism, as demonstrated in these experiments. If histamine is released in the allergic state as some contend (15) when antigen meets the histamine-antibody complex, the potential antihistaminic effect of Pyribenzamine is apparent.

Future reports will deal with the actions of Pyribenzamine on bronchial musculature, histamine wheals and anaphylactic hypotension.

CONCLUSIONS AND SUMMARY

1. The antagonistic properties of Pyribenzamine HCl, N′-pyridyl-N′-benzyl-N-dimethylethylene diamine HCl, against histamine phosphate have been described and discussed in relation to salivation, retraction of the nictitating membrane, lachrymation, mydriasis and blood pressure. Pyribenzamine, in the dosages employed, inhibited or obliterated the actions of histamine in all functions studied with the exception of mydriasis. The protection against histamine hypotension, however, was rather variable.

2. This antagonistic action of Pyribenzamine seems to have been specifically antihistaminic and not parasympatholytic or sympatholytic.

3. In fact, Pyribenzamine has the capacity in many instances to potentiate the responses of cholinergically and adrenergically controlled end-organs to their respective neural and humoral activators.

4. The import of Pyribenzamine's antihistaminic and simultaneous potentiating properties has been discussed in relation to clinical trial in allergic states.

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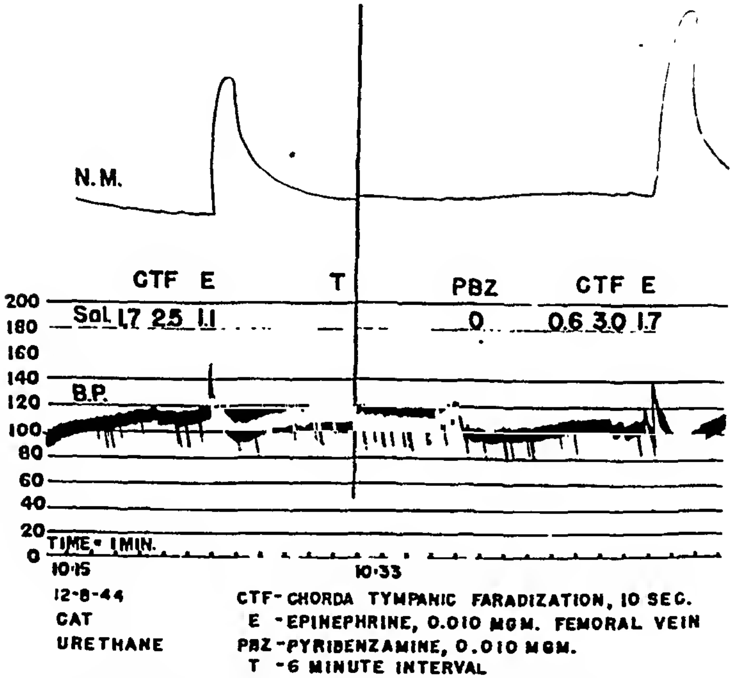

FIG. 7. 12/8/44. Cat, 2.67 kg., urethane anesthesia. From above downward: nictitating membrane, salivation, arterial pressure, time in minutes. Salivation after epinephrine, 0.010 mgm., was slightly increased after Pyribenzamine 0.010 mgm., and retraction of the nictitating membrane was markedly enhanced.

ergotoxine or yohimbine in that, as has been suggested, they may dampen certain enzymic systems associated with amine oxidase, phenolase, tyrosinase or other substances which normally may contribute to the destruction or degradation of epinephrine and sympathin or similar agents. Thus, Pyribenzamine may act in respect to sympathetic stimulation in a manner similar to the action of physostigmine in its relation to parasympathetic stimulation. This seems probable because of the concomitant parasympathetic sensitization observed in some of our experiments. Such increased cholinergic salivation has been observed

BARBITURATE ANTAGONISM OF ISONIPECAINE CONVULSIONS AND ISONIPECAINE POTENTIATION OF BARBITURATE DEPRESSION

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The increasing therapeutic use of isonipecaine (demerol or 1-methyl-4-phenyl isonipecotic acid ethyl ester hydrochloride) as an analgetic and spasmolytic agent suggests the need for a more thorough consideration of the toxicity of the compound. It was decided, therefore, to initiate a search for suitable agents which could antagonize isonipecaine overdosage.

Isonipecaine was recently reported to possess local anesthetic properties (1, 2). Its toxic actions as produced experimentally are also quite similar to those obtained with cocaine and procaine, being mainly those of central stimulation. Inasmuch as the barbiturates have been successfully used to antagonize the convulsive actions of certain local anesthetics (3, 4, 5), studies seemed indicated to ascertain whether or not they could act likewise in isonipecaine overdosage.

Method. Studies on toxicity were first made on white mice weighing approximately 20 grams. The minimal lethal dose (LD80) for isonipecaine subcutaneously and the intraperitoneal median lethal dose (LD50) of barbital, phenobarbital, amytal, pentobarbital, evipal and diphenylhydantoin (the sodium salts were used throughout) were respectively determined. In administering each compound the concentration of a given dose was adjusted so that each animal received not less than 0.2 cc. and not more than 0.6 cc. of a solution of the drug in distilled water. A fraction ($\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ or $\frac{3}{4}$) of the intraperitoneal LD50 of each barbiturate respectively was then injected in another series of mice and this was followed by a subcutaneous injection of the previously determined toxic dose (LD80) of isonipecaine. The number of survivals per number of animals was recorded and compared to the isonipecaine series which received no barbiturate.

In another experiment on white mice, after determining the minimal lethal dose (LD80) of isonipecaine intraperitoneally, a second group of animals was given the lethal dose of isonipecaine and then treated immediately with either barbital, phenobarbital, amytal, pentobarbital, evipal or diphenylhydantoin. As in the first experiment, the individual dose of each barbiturate administered was in fractions of its respective LD50. The treated series was compared with the untreated series as to survivals per number of animals at the LD80 of isonipecaine.

Studies were also made on white rabbits weighing 1.7 to 2.3 kilograms. The minimal lethal dose (subcutaneous LD80) was determined for isonipecaine as were the respective LD50s (intravenous) for phenobarbital, amytal, pentobarbital, evipal and diphenylhydantoin. The concentration of each anticonvulsant was adjusted so that between 5–10 cc. were injected in the ear vein at the rate of 5 cc per minute. Another group of rabbits was given the lethal subcutaneous dose of isonipecaine and this was immediately followed by an anticonvulsant intravenously, the dose of each compound being in fractions of its respective LD50. The percentage survivals of treated and untreated isonipecaine rabbits were recorded and compared.

The effects of the barbiturates ($\frac{1}{8}$–$\frac{1}{4}$ LD50) against isonipecaine intravenously and orally

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LD50) of a barbiturate was given beforehand, the percentage of animals surviving from the same dose of isonipecaine was 84%–90% (combined survival percentage of all the barbiturates). With the exception of amytal which was found to be most effective at $\frac{1}{16}$ LD50, the optimum single protective dose for a barbiturate was $\frac{1}{8}$–$\frac{1}{4}$ LD50. Diphenylhydantoin was ineffective in preventing lethal isonipecaine convulsions.

In another series, the barbiturates were also found to increase the intraperitoneal tolerated dosages of isonipecaine in mice (table 3), but its protective actions were not found to be as pronounced as against isonipecaine subcutaneously.

TABLE 3

Effect of anticonvulsants on the survival of white mice given a lethal dose of isonipecaine

TYPE OF BARBITURATE	BARBITURATE DOSAGE INTRAPERITONEALLY		ISONIPECAINE DOSAGE INTRAPERITONEALLY, MGM./KGM.				
	Fraction of LD50	mgm./kgm.	100	125	150	175	200
			Deaths/no. mice				
None (isonipecaine only) .			2/8	4/13	6/11	10/12	5/5
Phenobarbital..........	$\frac{1}{2}$	118				3/3	2/3
	$\frac{1}{4}$	59				6/13	4/4
	$\frac{1}{8}$	29				5/9	4/4
Barbital..	$\frac{1}{4}$	150				2/4	
	$\frac{1}{8}$	75				2/4	
Amytal	$\frac{1}{4}$	50				5/6	
	$\frac{1}{8}$	25				4/5	
	$\frac{1}{16}$	12.5				3/5	
Pentobarbital ..	$\frac{1}{4}$	30				4/9	
	$\frac{1}{8}$	15				4/8	
Evipal . .	$\frac{1}{4}$	68				4/7	2/2
	$\frac{1}{8}$	34				2/6	3/4
Diphenylhydantoin.	$\frac{1}{4}$	50				4/4	

Increasing the barbiturate dosage beyond the optimum anticonvulsant range ($\frac{1}{8}$–$\frac{1}{4}$ LD50) in mice which have received a lethal dose of isonipecaine does not increase the survival of animals (table 2). In fact, it was found that certain doses of barbiturates ($\frac{1}{2}$–$\frac{3}{4}$ LD50) ordinarily tolerated when given alone, hastened the death of animals which had also received a toxic dose of isonipecaine. No convulsions were noted and the animals died of respiratory depression shortly after the barbiturate was administered. Death seemed to occur sooner when a rapid acting barbiturate was employed.

The results on rabbits (table 4) substantiate the findings on mice. The in-

were also studied on a few rabbits. The method used was essentially similar to that described above. Five per cent isonipecaine was used for intravenous and oral administration, the intravenous injection rate being one cubic centimeter per minute.

RESULTS. Table 1 lists the median lethal dose (LD50) of the various compounds studied on mice and rabbits. From fifteen to fifty-eight animals were used for each determination. Rather high standard errors were obtained when

TABLE 1

Toxicity of the barbiturates, diphenylhydantoin* and isonipecaine*

COMPOUND	WHITE MICE		RABBITS	
	Route	LD_{50} with S.E.†	Route	LD_{50} with S.E.†
		mgm./kgm.		*mgm./kgm.*
Barbital	IP	620 ± 30.3		
Phenobarbital	IP	235 ± 11.6	IV	200 ± 4.3
Amytal	IP	200 ± 18.8	IV	75 ± 2.9
Pentobarbital	IP	123 ± 17.3	IV	40 ± 2.1
Evipal	IP	270 ± 17.0	IV	72 ± 3.6
Diphenylhydantoin	IP	190 ± 12.9	IV	59 ± 3.3
Isonipecaine‡	IP	145 ± 7.4		
	SQ	165 ± 6.9	SQ	200 ± 13.0

* The sodium salts.
† S.E. = standard error approximated according to Miller and Tainter (9).
‡ Intraperitoneal LD_{50} for Rats, 93 ± 4.1 mgm./kgm.

TABLE 2

Protective effects of barbiturates on white mice given a lethal subcutaneous dose of isonipecaine (200 mgm./kgm.)

ANTICONVULSANT	FRACTION OF ANTICONVULSANT LD_{50} (INTRAPERITONEALLY)					
	0 LD50 (isonipecaine only)	1/16 LD50	1/8 LD50	1/4 LD50	1/2 LD50	3/4 LD50
	Deaths/no. mice					
Phenobarbital	13/15		1/8	1/12	2/5	10/11
Barbital	13/15	3/4	1/4	0/3	1/4	
Amytal	13/15	0/4	1/3	2/4	3/4	
Pentobarbital	13/15		0/3	0/5	5/5	
Evipal	13/15		1/7	0/7	3/5	3/3
Diphenylhydantoin	13/15	3/4	4/4	4/4	3/4	

less than twenty animals were used. The median lethal doses of isonipecaine obtained on mice, rabbits and rats agree reasonably well with those previously reported (6, 7, 8).

Table 2 indicates that the barbiturates afford protection to mice given a lethal dose of isonipecaine. Only two out of fifteen mice (thirteen per cent) lived after a 200 mgm./kgm. dose of isonipecaine, whereas when a protective dose (1/8–1/4

when given isonipecaine, 150 mgm./kgm., subcutaneously, all convulsed and died, but no deaths occurred when seven rabbits, which had previously received phenobarbital ($\frac{1}{4}$ LD50), were respectively given isonipecaine, 150 mgm./kgm. (three animals), 175 mgm./kgm. (three animals) and 200 mgm./kgm. (one animal).

Barbiturate antagonism of oral isonipecaine overdosage was also studied on rabbits which were fasted twenty hours. An oral 400 mgm./kgm. dose of isonipecaine, which killed three out of three rabbits, was lethal only to one of three rabbits treated with phenobarbital ($\frac{1}{6}$ LD50). Attempts to protect twenty-one fasted animals from higher oral doses (600–900 mgm./kgm.) of isonipecaine with phenobarbital, amytal or evipal were not too successful. Although convulsions were prevented totally or in part and survival time sometimes prolonged several hours, the animals died eventually due to respiratory failure. However, two non-fasted rabbits survived an oral isonipecaine dose of 750 mgm./kgm. and had no convulsions.

Attempts were also made to combat intravenous isonipecaine overdosage with the barbiturates. In the few cases so tried, it was quite difficult to prevent fatalities because the safety margin of the barbiturates for their anticonvulsant effects and their causing respiratory depression in isonipecaine animals is quite small. When the minimal anticonvulsive dose of evipal ($\frac{1}{5}$ LD50) was used, convulsions and sometimes fatalities were prevented, but just as often the animals would die of respiratory depression.

As with mice, it was found (table 4) that when the dose of the barbiturate in rabbits was increased to about $\frac{1}{2}$ to $\frac{3}{4}$ of its LD50 for antagonizing isonipecaine overdosage, the animals died without convulsing in respiratory depression and their survival time was less than that of animals which had received a lethal dose of isonipecaine only. As to be expected the short acting barbiturates caused death the most rapidly. Subsequently it was also found that even when the amount of isonipecaine was reduced to about $\frac{1}{8}$ its subcutaneous LD50 (25 mgm./kgm.), respiratory failure resulted in rabbits which had previously received an ordinarily tolerated barbiturate dosage ($\frac{3}{4}$ LD50). Also the administration of $\frac{1}{2}$ LD50 of isonipecaine and $\frac{1}{2}$ LD50 of a barbiturate caused a greater percentage of deaths than could be expected from the additive effects of each compound. Surviving animals so treated remained depressed for a considerably longer period than rabbits which had received the same dose of a barbiturate only.

Discussion. The toxic signs of isonipecaine overdosage as noted in mice, rats and rabbits are quite similar, being increased excitability, dyspnea, Cheyne-Stokes respiration, clonic convulsions and opisthotonos with possible death due to respiratory failure. From the results it appears that any barbiturate should antagonize these convulsive symptoms, and as judged from the minimal anticonvulsant dose of each barbiturate, no one barbiturate possesses appreciable superiority over the others for suppressing isonipecaine convulsions. Although our results on rabbits seem to favor phenobarbital as the most effective agent, this seeming superiority of phenobarbital is actually due in part to experimentally imposed conditions. It is rather to be expected that when a single dose of a

travenous injection of a barbiturate (¼ LD50) in animals, which had previously received a lethal convulsive dose of isonipecaine (250 mgm./kgm., subcutaneously), aborted convulsions and in many instances prevented fatalities. Phenobarbital gave the highest survival percentage but animals so treated had several isonipecaine convulsions before the effects of phenobarbital became evident. Diphenylhydantoin was ineffective in the dosage employed (¾, ½, ¼ LD50). Most of the barbiturates with the exception of phenobarbital, when administered in a single dose (¼ LD50), prevented isonipecaine convulsions for one or two

TABLE 4

One way barbiturate-isonipecaine antagonism in rabbits

(Results recorded as deaths/number of animals)

ANTICONVULSANT	FRACTION INTRAVENOUS LD50	ISONIPECAINE DOSAGE, MGM./KGM. (SUBCUTANEOUSLY)								
		25	50	100	150	175	200	250	300	400
No barbiturate ..					0/4	1/4	2/4	4/4		
Phenobarbital	¾	2/2*	1/1*						3/3*	3/3*
	½		1/2*				1/2*		1/3*	3/3*
	¼				0/3	0/2	0/2		0/3	2/2†
	⅛								0/3	
	1/16								4/4†	4/4†
Amytal	½			2/2*					2/2*	
	¼							1/3†	2/3†	
Pentobarbital	¾						3/3*		1/1*	
	½						2/2*		3/3*	
	¼						0/4		1/3†	
Evipal	¾	3/4*	1/1*	1/1*						
	½		1/3*	4/4*						
	¼							3/3†	2/2†	
Diphenylhydantoin	¾			0/2						
	½							2/2		
	¼							2/2		

* Animals died of respiratory depression shortly after barbiturate injected.

† Animals showed some protection but died following convulsions.

hours, but the rabbits died later with recurrence of convulsive symptoms. Subsequently it was found that repeated injections of evipal whenever necessary prevented the deaths of three rabbits which had received 300 mgm./kgm. of isonipecaine subcutaneously.

Since it is generally known that older rabbits may sometimes differ from younger rabbits in their responses to analeptic and depressant compounds, the effects of the barbiturates on isonipecaine overdosage were also investigated on a few older rabbits weighing approximately three kilograms. Three older rabbits,

it is conceivable that cases of isonipecaine poisoning may result from excessive doses taken accidentally or with suicidal intent. The question then arises as to the efficacy of using a barbiturate to combat any resulting toxic central stimulatory action. Treating such cases during the early stages of isonipecaine poisoning may prove to be of value, but it represents a rather hazardous undertaking because—as has been pointed out—the margin of safety for such a procedure extends over a rather narrow range which becomes even narrower with increasing amounts of isonipecaine in the body. Should the treatment be attempted, the barbiturate of choice should probably be one with an intermediate duration of action such as pentobarbital or amytal. Such agents may be expected to have a slightly delayed onset of action in producing respiratory depression, in which action isonipecaine may potentiate, and yet act with sufficient rapidity on the higher centers so as not to defeat the purpose of its anticonvulsive usage. Just enough barbiturate should be cautiously administered to decrease the severity of the convulsions. Attempts should not be made to block convulsions entirely in order to minimize the possibility of causing respiratory depression.

These findings on isonipecaine substantiate our previous reports (2, 13) that isonipecaine has certain properties in common with cocaine in addition to its established similarities to morphine and atropine (6, 7, 8, 13). In line with our conclusion are the reports that cocaine in large doses enhances pentobarbital narcosis and may prolong recovery time (14), and that its antidotal effect for barbiturate poisoning is unpredictable, being analeptic in some cases and hastening respiratory failure in others (15). It would seem, therefore, that the relationship between the barbiturate and cocaine on one hand, and with isonipecaine on the other, is qualitatively similar, with isonipecaine giving the greater additive effect on barbital depression and lesser antidotal effect against barbital depression than cocaine.

These findings on isonipecaine also serve to bring out further the similarity in action of isonipecaine to morphine, the latter compound being also biphasic in its central actions (16). We believe, therefore, that the claims that isonipecaine causes little or no respiratory depression, especially when it is used in preanesthetic medication, should be considered more carefully. At any rate, a comparative study of the potentiating properties of morphine and isonipecaine on the toxicity of hypnotics and anesthetics is indicated.

ACKNOWLEDGEMENTS. The Department of Medical Research of Winthrop Chemical Company made possible the completion of this investigation by furnishing a grant and a supply of isonipecaine and evipal. Mr Rollan Swanson rendered important technical assistance. Professors L W. Hazleton and E. William Ligon, Jr. gave valuable advice and timely suggestions.

SUMMARY

1. It was found that barbital, phenobarbital, amytal, pentobarbital, and evipal ($\frac{1}{8}$ to $\frac{1}{4}$ LD50 of the sodium salts) aborted convulsions and usually prevented death in mice and rabbits given an ordinarily lethal dose of isonipecaine.

depressant is used to counteract a rather long acting convulsant, phenobarbital should give the greatest protection by simply affording the body more time to dispose of the convulsive agent. That the short acting barbiturates are quite effective is indicated by our other experiments in which we were able to prevent death in rabbits given ordinarily lethal doses of isonipecaine by injecting repeatedly the ultra-short acting evipal as required.

In mice, however, the fact that a simple optimum dose of any barbiturate is usually sufficient to prevent lethal isonipecaine convulsions is probably due to isonipecaine's being more rapidly detoxified in mice than in rabbits, thus allowing the short acting barbiturates to have an adequate duration of effect for preventing isonipecaine fatalities. This conclusion is based on the observation that mice seem to recover more rapidly than rabbits from the effects of convulsive but non-lethal doses of isonipecaine. However, the fact that the barbiturates were administered intraperitoneally in mice but intravenously in rabbits also accounts in part of the better results obtained on mice with the short acting barbiturates.

It is evident from the results that there is a distinct limitation on use of barbiturates for isonipecaine overdosage. Large doses of barbiturates ordinarily tolerated may cause respiratory depression in animals which have also received isonipecaine. This finding is supported by reports that isonipecaine potentiates the hypnotic properties of evipal (10) and seconal (11), and by the fact that it also shortens the induction time and prolongs the duration of effect of certain anesthetic agents (12).

The inability of isonipecaine to antagonize the barbiturates plus its additive effect on barbiturate depression may be explained from the results of various investigators (6, 7, 8) who have reported that isonipecaine depresses respiration in anesthetized animals. Duguid (7) explains the central nervous effects of isonipecaine to be "cortical stimulation followed by depression, together with a primary depression of the medulla." It is probable, therefore, that the use of isonipecaine after a large dose of a barbiturate serves to intensify the depressant action of the barbiturate on the medulla. The loss of our animals so treated can be explained on this basis. On the other hand, the early convulsive deaths that occur in animals overdosed with isonipecaine may be prevented by suppressing convulsions with a minimal anticonvulsive dose of a barbiturate. However, the protective effects of the barbiturates are limited in turn by the amount of isonipecaine present in the animal because the depressant effects of isonipecaine probably become more evident at high doses, so that even though convulsions are prevented, the animals die due to isonipecaine potentiation of barbiturate depression. The less favorable results obtained with barbiturates for intravenous and intraperitoneal isonipecaine overdosage are also in line with this conclusion.

In man the chief toxic signs of isonipecaine overdosage are those of cerebral irritation. Severe tremors and uncoordinated muscular movements appear, and occasionally epileptiform seizures develop (17, 18). Although no convulsions have been reported from the administration of isonipecaine in therapeutic doses,

THE BLOCKING EFFECT OF TETRAETHYLAMMONIUM ION ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

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In a previous paper (1) tetraethylammonium ion was shown to have a vasodepressor effect in anesthetized cats and dogs. The data presented there led to the conclusion that this response is due to a block of the vasoconstrictor discharge from the spinal cord and that this block occurs at the sympathetic ganglia, at which the preganglionic vasoconstrictor nerves make synaptic connections with postganglionic neurons. Evidence was adduced that the cardiodecelerator response which usually accompanies the vasodepressor response is due to a ganglionic block in the pathway of the cardioaccelerator fibers.

The present report deals with studies of the effects of tetraethylammonium ion on the superior cervical ganglion of the cat, undertaken in an attempt to analyze the mechanism of action.

METHOD. Cats were used under anesthesia with 0.7 cc. per kgm of Dial solution[2] (each cc. containing diallyl barbituric acid 0.1 gram, urethane 0.4 gram, monoethyl urea 0.4 gram, in aqueous solution) injected intraperitoneally. A tracheal cannula was inserted. The cervical sympathetic chain on one side of the neck was dissected. The vagus nerve was cut at some distance cephalad from the electrodes. The vagus and the cervical sympathetic nerve were crushed lower in the neck. When postganglionic fibers were stimulated, cranial nerves IX, X, XI, and XII were dissected and removed away from the electrodes. In some experiments the superior cervical ganglion was crushed. The preganglionic or postganglionic nerves were stimulated maximally through shielded, platinum electrodes with biphasic discharges.

The eyelids were retracted after a cut into the lateral canthus. The nictitating membrane was connected to an isotonic lever magnifying its movements 14 to 28 times and recording them on a smoked drum.

Intravenous injections were made through a cannula inserted into the femoral vein and were flushed into the blood stream with 2 to 3 cc. of saline. Intra-arterial injections were made in a volume of 0.1 cc. into the external carotid artery through a needle inserted into the lingual artery. When intra-arterial injections were made, the external carotid artery was ligated cephalad to the branching off of the lingual artery.

Tetraethylammonium bromide (Eastman Kodak Company) was used in all experiments and was made up in an aqueous stock solution of 10% strength and diluted appropriately shortly before injection. Elementary analysis and tests for ash and amines showed that the sample of tetraethylammonium bromide used was pure.[3] Intocostrin[4] was used as a standardized curare preparation.

RESULTS. *A. Effect on contractions of nictitating membrane produced by preganglionic stimulation.* Stimulation of the preganglionic fibers of the nictitating

[1] Fellow of the Rockefeller Foundation.

[2] Generously supplied by Ciba Pharmaceutical Products, Summit, New Jersey.

[3] Tests done by Dr. Carl Tiedeke, New York.

[4] Generously supplied by E. R. Squibb and Sons, New York.

2. When the dose of each barbiturate was increased to about $\frac{1}{2}$ or $\frac{3}{4}$ of its respective LD50 for antagonizing isonipecaine overdosage, it was found that the animals died of respiratory depression, convulsions did not precede death. Subsequently it was found that even when the amount of isonipecaine was reduced to about $\frac{1}{8}$ its LD50, respiratory failure resulted in animals which had previously received an ordinarily tolerated barbiturate dosage ($\frac{3}{4}$ LD50).

3. Diphenylhydantoin (sodium salt) did not act like the barbiturates in the above respects

4. It is concluded that isonipecaine barbiturate antagonism acts only in one direction. The barbiturates antagonize the lethal convulsive effects of isonipecaine, but isonipecaine potentiates the depressive properties of the barbiturates on respiration.

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still produced a response of the membrane (fig. 4). When the injections of potassium chloride were repeated after the superior cervical ganglion had been crushed, no response of the nictitating membrane occurred (fig. 3).

E. Comparison of tetraethylammonium ion and curare. The administration of curare (Intocostrin) during preganglionic nerve stimulation produces temporary relaxation of the nictitating membrane similar in many respects to that produced by tetraethylammonium ion. The amplitude and duration of the relaxation vary with the dose of curare in a manner similar to that described for tetraethylammonium ion.

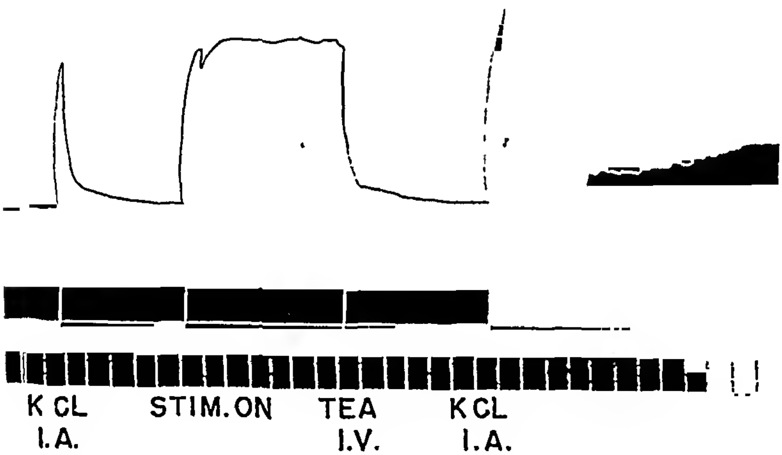

FIG. 4. RESPONSE TO POTASSIUM ION DURING COMPLETE BLOCK OF PREGANGLIONIC IMPULSES BY TETRAETHYLAMMONIUM ION. CONTRACTIONS OF NICTITATING MEMBRANE

At signals K Cl, intra arterial injection of potassium chloride, 5 mgm. At STIM. ON, beginning of maximal preganglionic stimulation at 3 per sec. At TEA, intravenous injection of tetraethylammonium bromide, 10 mgm. per kgm. Time in minutes

Certain differences between the action of curare and that of tetraethylammonium ion are apparent. Small doses of curare injected intra-arterially during preganglionic stimulation usually caused a preliminary contraction of the nictitating membrane, an effect not observed with tetraethylammonium ion. The relaxation produced by curare was slower to reach its maximum and lasted longer for a given degree of relaxation.

The curare used (Intocostrin) is biologically standardized, and prepared so that 1 mgm. of the material contains a uniform curarizing potency. The smallest dose which upon intra-arterial injection would produce a relaxation of the nictitating membrane stimulated via its preganglionic nerve was 0.25 microgram. Complete relaxation of the membrane occurred with 0.5 mgm. When injected

doses was followed by quick and complete recovery. When tetraethylammonium bromide was injected intravenously, only doses of 5.0 mgm. per kgm. or larger decreased the response to acetylcholine, 10 mgm. per kgm. causing abolition of the response. Recovery occurred in from 5 to more than 60 minutes, depending on the dose of tetraethylammonium bromide. When the direct action of acetylcholine on the nictitating membrane was eliminated by the intravenous administration of atropine sulfate (1 mgm. per kgm.), intra-arterial injections of acetylcholine still produced contractions of the nictitating membrane. In 3 experiments this effect was decreased by tetraethylammonium ion (0.1 to 1.0 mgm. intra-arterially or 10 mgm. per kgm. intravenously).

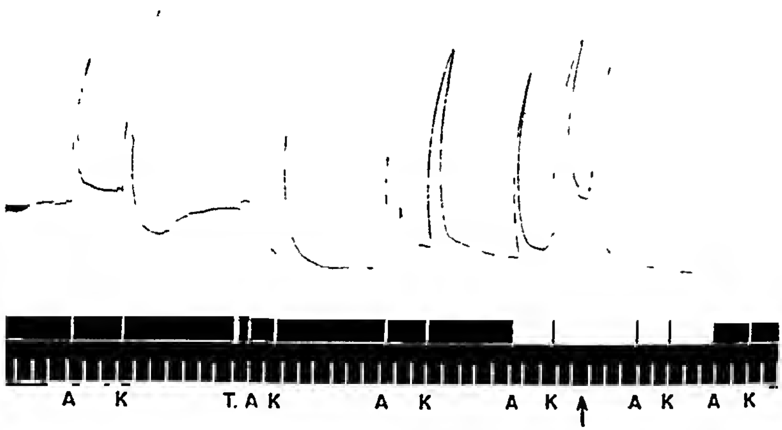

FIG. 3. EFFECT OF TETRAETHYLAMMONIUM ION ON GANGLIONIC RESPONSES TO ACETYLCHOLINE AND POTASSIUM ION CONTRACTIONS OF NICTITATING MEMBRANE

At signals A, intra-arterial injection (via lingual artery into carotid, the external carotid being ligated beyond the branching off of the lingual) of acetylcholine bromide, 0.1 mgm. At signals K, intra-arterial injections of potassium chloride, 1 mgm. At signal T, intravenous injection of tetraethylammonium bromide, 10 mgm per kgm. At the arrow, the superior cervical ganglion was crushed Time in minutes.

In 3 experiments, intra-arterial acetlycholine elicited responses of the nictitating membrane after the superior cervical ganglion had been crushed (and in the absence of atropine). This action of acetylcholine was not affected by tetraethylammonium bromide even when the latter was injected in doses as large as 10 mgm. intra-arterially.

D. Effect on responses to potassium ion. Potassium chloride, injected intra-arterially in doses of 1.0 or 5.0 mgm., elicited contraction of the nictitating membrane. Tetraethylammonium bromide injected intravenously in doses of 10 mgm. per kgm. did not cause a decrease of the response to potassium ion (fig. 3). When the effects of preganglionic impulses were completely blocked by tetraethylammonium bromide, intra-arterial injections of potassium chloride

tassium ion stimulated a part of the ganglion itself, rather than postganglionic structures, is indicated by the absence of any response of the nictitating membrane after the ganglion had been crushed. Thus the potassium-sensitive part of the postganglionic neuron is not affected by tetraethylammonium ion.

The blocking action of tetraethylammonium ion at the superior cervical ganglion is similar to that of curare. When Intocostrin was compared with tetraethylammonium ion with respect to the ganglionic action, relatively slight differences between the two substances were observed. The curare preparation is the more potent of the two and produces effects which reach their maxima later and last longer. Yet the curare preparation in small doses increases the discharge of the ganglion, a phenomenon not observed with tetraethylammonium ion. A similar effect (increase of twitch height) is seen in skeletal muscle not only with curare (5) but also with tetraethylammonium ion (4).

In the superior cervical ganglion tetraethylammonium ion, in contrast to curare and other substances, has a pure blocking action, with no trace of stimulation, either with small doses or with large doses. The slight contractions of the nictitating membrane which were sometimes produced by large doses of tetraethylammonium ion occurred as often when the ganglion was crushed as when it was intact. Hence these contractions cannot be attributed to a ganglionic stimulation by this substance, but may result from stimulation of the apparatus which lies peripheral to the ganglion. A parallel phenomenon, vasoconstriction, occurs when tetraethylammonium ion is injected into the femoral artery of the dog (1). Vasoconstriction has also been noted in the perfused hind leg preparation of the frog (6). One of two possible explanations of these stimulatory phenomena seems likely. Tetraethylammonium ion stimulates either the postganglionic axons of the nictitating membrane of the cat and the vasoconstrictor nerves in the dog and frog, or else these smooth muscles themselves. There is evidence that the substance may stimulate nerve axons (7). Evidence on denervated smooth muscles is lacking The important points for the present discussion are that these stimulatory actions of tetraethylammonium ion are not on the ganglion and that they are at best slight and inconsistent

The data presented in this paper strengthen the conclusions previously made that tetraethylammonium ion does not possess most of the pharmacological properties characteristic of the other quaternary ammonium compounds (1). Tetraethylammonium ion has, if any, only slight and inconstant muscarinic effects and no atropine-like effects on the nictitating membrane. And it has no "nicotinic stimulating" effects on the superior cervical ganglion. Although it is quite similar to curare in blocking the transmission of impulses through autonomic ganglia, it has little, if any, curarizing action in skeletal muscle (8, 4).

The one predominant effect of tetraethylammonium ion is the block of transmission across autonomic ganglia According to the terminology currently in use, this block would be designated a "nicotinic paralyzing" effect. For not only the effects of acetylcholine, but also those of preganglionic nerve impulses are blocked at the organ which is most closely associated with "nicotinic" effects, that is, the autonomic ganglion. Yet the action of tetraethylammonium ion

intravenously, 0.03 and 1.2 mgm. per kgm. of Intocostrin produced, respectively, minimal and complete relaxation of the nictitating membrane.

When doses of the order of one to ten micrograms of Intocostrin or tetraethylammonium bromide were injected intra-arterially, the relaxation of the nictitating membrane was of measurable amplitude and of short enough duration so that a series of observations could be made. In three such experiments, attempts were made to compare the potency of Intocostrin and of tetraethylammonium bromide. Approximately 2 to 5 times as much tetraethylammonium bromide was necessary to produce a relaxation of the same magnitude as that produced by a given dose of Intocostrin.

During the relaxation produced by curare, injections of tetraethylammonium bromide always caused a further relaxation. No decurarizing effect, such as tetraethylammonium ion produces in skeletal muscle (4) was observed.

When curare was injected intravenously during stimulation of the postganglionic nerve after destruction of the superior cervical ganglion, the contractions of the nictitating membrane were unaffected, even though the dose of curare was as great as 10 mgm. per kgm.

Discussion. When tetraethylammonium ion is administered during stimulation of the cervical sympathetic nerve, the nictitating membrane undergoes a temporary relaxation. The data presented above permit the localization of the site of this action at the superior cervical ganglion. Tetraethylammonium ion does not block the stimulating action of epinephrine or acetylcholine on the nictitating membrane itself. Nor does it affect the responses of the nictitating membrane to stimulation of the postganglionic axons. Hence the neuromuscular system peripheral to the ganglion is not affected by this substance. The integrity of the preganglionic axons was not directly tested. Yet there is no block of conduction in postganglionic axons. Furthermore, the impulses in motor nerves to skeletal muscle are not blocked by a wide range of doses of tetraethylammonium ion (4). We may therefore assume that the impulses in the preganglionic axons reach the ganglion. Since we know that impulses set up immediately beyond the ganglion are not blocked, we conclude that the ganglion is the site of action of tetraethylammonium ion.

The stimulating effect of acetylcholine on the superior cervical ganglion is also blocked by tetraethylammonium ion. This fact is consistent with the theory that tetraethylammonium ion blocks ganglionic transmission by preventing the acetylcholine released at the preganglionic nerve endings from stimulating the postganglionic neurons. We have no evidence as to whether or not tetraethylammonium ion affects the release of acetylcholine from cholinergic nerve fibers. Blockage of the effects of injected acetylcholine, however, accounts adequately for the block of the effects of preganglionic impulses.

The alternative possibility that tetraethylammonium ion produces a ganglionic block by rendering the postganglionic neurons inexcitable was tested by the use of potassium chloride. Even during complete block of the stimulating action of acetylcholine or preganglionic impulses, potassium ion produces a characteristic stimulation of the postganglionic neurons. That in these experiments the po-

THE PHARMACOLOGY OF DI-ISOPROPYL FLUOROPHOSPHATE (DFP) IN MAN

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Extensive pharmacological and biochemical studies have been carried out on the effect of di-isopropyl fluorophosphate (DFP) upon various laboratory animals (1) (2). It has been found to act predominantly or solely, in the doses employed, by inactivation of the enzyme cholinesterase (Ch.E.). By comparison with other known anticholinesterase agents, its action is prolonged, lasting for days or weeks. This fact coupled with results obtained from *in vitro* experiments has led to the interesting conclusion that DFP irreversibly destroys cholinesterase rather than temporarily inactivating it by the formation of a reversible combination. Consequently, after the administration of DFP, Ch.E. activity appears to be restored only by the synthesis of new enzyme in the plasma, red blood cells and tissue cells.

Though chemically quite dissimilar, the actions of DFP are qualitatively similar in most respects to those of neostigmine and eserine. It was obvious that DFP deserved a clinical trial in conditions such as myasthenia gravis and glaucoma in which a marked prolongation of an eserine-like action might be desirable therapeutically. Consequently, the pharmacology of DFP in man has been studied in normal subjects and in patients with mysthenia gravis and glaucoma. The effectiveness of DFP in the treatment of these diseases and special phases of its ocular pharmacology are being reported elsewhere (3) (16).

METHODS. DFP is a colorless liquid with a specific gravity of 1.055 and a solubility of 1.54% in water at 25°C. It was prepared for clinical use as a 0.1% solution in peanut oil. Two cc. aliquots were placed in glass ampoules and sterilized by autoclaving at 117°C. for 60 minutes. It has been shown that DFP so prepared maintains its potency for at least one year, but if diluted with water, it loses its potency within a few days. When aqueous solutions were used, these were prepared immediately before intramuscular use employing non-autoclaved DFP, sterile water and syringes and sterile technique. No evidence of local irritation was ever noted following intramuscular injections. For oral administration, a 1% solution of DFP in peanut oil was placed in gelatin capsules of convenient size.

The action of the drug was studied in 7 normal subjects, 8 patients with no organic disease, and 7 patients with myasthenia gravis.

Cholinesterase determinations were carried out upon plasma and RBC from samples of heparinized venous blood by a modification of the Ammon method as described by Mazur and Bodansky (1) The method measures the rate of liberation of CO_2 over a 30 minute period in the Warburg apparatus from a $NaHCO_3$-acetyl choline bromide solution. Results were calculated as μM CO_2 liberated from 1 cc. of a 1:5 dilution of plasma or 1:10 dilution of RBC hemolysate. Control values were determined on samples taken from each individual before the administration of DFP and at least 12 hours after the last dose of neostigmine (if the latter had been administered previously); subsequent values are expressed as percent-

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has little resemblance to that of nicotine. It lacks completely the stimulating property and all the other properties of nictoine except the power to block ganglia. If pharmacological effects are to be named after pharmacological agents, it would seem that the blockage of ganglia should be named after the substance which in the purest manner exemplifies this effect. A more satisfactory alternative is, however, to describe the action of the pharmacological agent in terms of the site and nature of its action. Tetraethylammonium ion, then, over a wide range of doses exerts a specific ganglionic blocking action.

SUMMARY

In cats under dial anesthesia the effects of tetraethylammonium bromide were studied on the nictitating membrane and its innervation. This substance does not materially affect the membrane itself or its postganglionic nerves. It produces a block of transmission of nerve impulses across the superior cervical ganglion. The ganglionic stimulating action of acetylcholine is also blocked, but that of potassium ion is not. Unlike curare, many other quaternary ammonium compounds, and nicotine, tetraethylammonium ion has no actions except this specific ganglionic blocking action over a wide range of doses.

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ages of the controls. It should be pointed out that because of the irreversibility of the DFP-Ch.E. complex, such factors as dilution (which necessitates complicated corrections in the determination of Ch.E. activity following neostigmine (4)) do not interfere with Ch.E. determinations following DFP.

Liver function was studied in the patients with myasthenia gravis by the following tests: plasma bilirubin (5), cephalin-cholesterol flocculation (6), bromsulfalein excretion (7), Watson urobilinogen (8), Wallace and Diamond urobilinogen (9), Harrison bilirubin spot (10), methylene blue (11), and blood cholesterol (12). Kidney function was evaluated by determinations of urinary specific gravity, blood urea nitrogen, PSP function test, and van Slyke urea clearance test. Determinations of red blood cell count, hemoglobin, white blood cell count, differential white cell count, blood sugar, and plasma proteins were also done at intervals during and after the course of therapy. The effect upon the cardiovascular system was determined by injecting 2.0 mg. of DFP in oil intramuscularly into 7 normal fasting adults. Pulse, blood pressure, cardiac output (ballisto-cardiogram) and ECG were recorded during the control period, and at intervals of 1, 2, 5, 9, and 12 hours after injection. The ballisto-cardiograms were measured by the area method (13). The subjects were either resting continuously, or had a complete rest of 30–60 minutes before recording. Inasmuch as the cardiovascular system becomes "basal" after the BMR has reached its minimum, a patient was not considered basal until a steady pulse had been obtained over a 30 minute period. Blood sugar was measured by a modification of the Benedict method (14) before and one to two hours after DFP in order to detect any stimulation of the adrenal medulla following DFP. Vital capacity measurements were made in 5 of the subjects to determine the possible occurrence of bronchoconstriction; the best of three trials was recorded.

RESULTS. *A. The Effect of DFP upon blood Ch.E. activity.* In table 1 are shown the reductions in plasma and RBC Ch.E. activity following the initial oral or intramuscular administration of DFP. This confirms the results previously reported that plasma Ch.E. is very sensitive to the action of DFP and may be depressed, to very low levels, without any significant reduction in the activity of erythrocyte Ch.E. (1) (2). It can be seen that in the higher dosage range, the plasma Ch.E. activity was lowered to 1–25% of its original value while the RBC Ch.E. was reduced to 62–94% of its control value.

This table also indicates that comparable lowering of RBC Ch.E. was usually accomplished by administration of 8 to 10 mg. of DFP in oil orally and by 2 to 3 mg. of DFP in oil intramuscularly. In the lower dosage ranges at which RBC Ch.E. was not affected, comparable reductions in plasma Ch.E. followed administration of 2 mg. orally and 0.5 intramuscularly. This confirms the results obtained in experiments upon monkeys (2) that the oral dose is approximately 3–5 times that of the intramuscular dose.

The rate of absorption of DFP after intramuscular and oral administration was followed by measuring, at frequent intervals, its effect upon blood Ch.E. activity. In 2 subjects (H. G. and S. C., table 2) the RBC Ch.E. reached its lowest level within three hours after intramuscular injection of 2 mg. of DFP in oil. In one subject (R. W.) the RBC Ch.E. reached its minimum activity within 43 minutes, after injection of 2 mg. of an aqueous solution of DFP. In our experience, though the absorption rates differ, the amount absorbed is equal from both vehicles following intramuscular injection. In 7 other patients, blood samples were taken $4\frac{1}{2}$ and 24 hours after oral administration of DFP in oil; in 5 of this group, minimal values for RBC Ch.E. were obtained in the $4\frac{1}{2}$ hour

TABLE 1

Ch.E. levels in plasma and RBC hemolysate after initial administration of DFP

PATIENT	WEIGHT	DFP	ROUTE	% OF CONTROL Ch.E. ACTIVITY	
				Plasma	RBC
	kg.	mg.			
F. P.	85	0.7	i.m.	40	110
M. C.	87	0.7	i.m.	35	102
B. M.	95	0.8	i.m.	42	104
S. C.	58	2.0	i.m.	2	77
S. C.[2]	58	2.0	i.m.	10	64
H. G.	67	2.0	i.m.	10	84
N. A.	43	2.5	i.m.	6	69
R. G.	49	2.5	i.m.	6	75
A. D.	42	3.0	i.m.	2	70
R. W.	41	3.0	i.m.	9	70
L. G.	60	3.0	i.m.	1	63
R. B.	82	2.8	oral	57	96
G. K.	76	2.5	oral	26	108
A. G.	61	2.0	oral	25	107
W. McN.	59	6.0	oral	2	89
H. W.	58	6.0	oral	7	87
J. C.	68	8.0	oral	1	94
H. M.	82	8.0	oral	25	84
A. B.	48	8.0	oral	2	62
L. H.	52	8.0	oral	1	63
A. S.	61	10.0	oral	1	66
E. M.	56	10.0	oral	2	67

TABLE 2

Rate of inactivation of Ch.E. following DFP intramuscularly

PATIENT	DOSE	TIME AFTER ADMINISTRATION	Ch.E. ACTIVITY	
			Plasma	RBC
	mg.			
H. G.	2.0 (oil)	Control	100%	100%
		1 hr.	34	91
		2 hrs.	33	94
		3 hrs.	10	86
		5 hrs.	11	84
S. C.	2.0 (oil)	Control	100%	100%
		1 hr.	2	93
		2 hrs.	2	79
		3 hrs.	2	
		19 hrs.	13	77
R. W.	2.0 (water)	Control	8%*	66%
		43 min.	2	40
		63 min.	2	41

* In those instances in tables 2 and 3, in which the control Ch.E. values are less than 100%, DFP had been given previously.

samples. In another patient, blood samples were taken at hourly intervals; analysis of these indicated that complete absorption occurred within 3 hours.

Other investigators (1) (2) have noted that RBC Ch.E. activity in animals returns to normal much more slowly than does plasma Ch.E. Our studies have confirmed this finding in man. As shown in table 3, plasma Ch.E. activity began to increase within 24 hours after the last dose of DFP while RBC Ch.E. rarely showed any significant increase within 3 days. In H. G., plasma Ch.E. rose from 2 to 94% in 19 days while RBC Ch.E. increased from 44 to only 50% over the same period.

These results confirm the conclusion reached in animal experiments (2) that determination of plasma and red cell Ch.E. activity are of no value as a guide to clinical dosage. Plasma Ch.E. was often reduced to 1% of its original value by a single dose of DFP without any detectable signs or symptoms of increased parasympathetic activity. The red cell Ch.E. should be a more accurate indication of the degree of inhibition of this enzyme in nerve and muscle, since the sensitivity of RBC Ch.E. to DFP more closely approximates that of muscle and nerve (1). However, subjects often noted marked side effects (see below) following single doses of DFP when the RBC Ch.E. was 70–75% of its original activity, and later, after repeated dosage, had fewer or no symptoms even though the RBC Ch.E. was below 15%.

B. Symptoms produced by DFP. The reactions following the initial administration of DFP (2 to 3 mg. intramuscularly or 6 to 10 mg. orally) to 22 individuals were carefully noted. Gastrointestinal symptoms were most common. There were 12 complaints of nausea, 10 of epigastric distress, "indigestion" or belching, 7 of anorexia, 4 of vomiting, 2 of abdominal cramps, 2 of diarrhea and 3 of increased peristalsis. The gastrointestinal tract was observed fluoroscopically in 3 patients with myasthenia gravis at the height of the nausea produced by DFP. In one (R. W.) the stomach emptied more slowly than normally ("extreme hypotonia") and regurgitation of the contents of the duodenum was noted. In a second (A. D.) there was slight difficulty in the initiation of the swallowing act. In the third (S. C.) the stomach emptied slowly, but its contents could be pushed easily into the duodenum; an 00 capsule was temporarily arrested at the level of the aortic arch but there was no evidence of cardiospasm.

In some subjects there were complaints that may be referable to the central nervous system: 4 of dizziness, 2 of shakiness, 3 of weakness, 2 of unusual number and character of dreams or nightmares, and 2 of a peculiar "drifting" sensation upon dozing. Three patients complained of substernal tightness and 2 of "choking sensations" which may have been due to bronchoconstriction. Four subjects complained of back pain.

Secretory activity was not measured objectively following administration of DFP, but no subject complained of increased sweating or salivation. In two female patients, dribbling of urine was noted occasionally; a similar occurrence followed the use of neostigmine in these patients. This was probably due to the relaxation of the bladder sphincter brought out by parasympathetic stimulation.

The six patients with myasthenia gravis who received the drug repeatedly

TABLE 3

Rate of regeneration of Ch E. following intramuscular administration of DFP

PATIENT	DOSE	TIME AFTER LAST DOSE	Ch.E ACTIVITY	
			Plasma	RBC
	mg			
H. G.	2.0 (oil)	Control	12%*	70%
		3 hrs	2	44
		8 hrs.	4	37
		29 hrs.	11	39
		53 hrs.	26	42
		3 days	31	42
		4 days	40	44
		5 days	46	
		6 days	50	42
		7 days	45	43
		8 days	52	44
		10 days	67	49
		16 days	81	46
		19 days	94	50
		22 days	124	50
		25 days	122	50
L G.	3.0 (water)	Control	100%	100%
		80 min.	0	76
		22 hrs	15	63
		46 hrs	34	61
		72 hrs.	42	71
S C	3 0 (oil)	Control	46%	56%
		4 hrs	1	33
		3 days	30	34
		4 days	35	48
		5 days	43	43
S C.	3 0 (oil)	Control	43%	43%
		4 hrs	3	22
		28 hrs	10	19
		2 days	16	25
		4 days	30	22
		6 days	39	25
A. D.	2 0 (oil)	Control	23%	61%
		40 hrs	17	51
		64 hrs	25	44
		88 hrs	36	45
		112 hrs	39	45
A D.	3.0 (oil)	Control	33%	30%
		17 hrs.	5	23
		62 hrs	14	24
		86 hrs.	26	21
		110 hrs	37	28

* See note, table 2.

noted the above symptoms on numerous occasions. In addition 3 patients complained of chilly sensations, 3 of headache, 2 of palpitation, 2 of paraesthesias, 3 of muscle twitching or cramps and one of "strained eye." Despite its marked effect upon the eye when instilled locally (3), no evidence of definite miosis or of ciliary spasm was observed following systemic administration.

In 6 patients with myasthenia gravis, a comparison was made of the side actions produced by neostigmine and DFP. The doses of neostigmine used were sufficient to control the symptoms of myasthenia; the doses of DFP were such that symptoms were only partially controlled. As shown in table 4, nausea was produced by DFP in 45% of 49 administrations, while it followed the use of neostigmine in only 3.5% of 308 administrations. This occurred despite the fact that repeared doses of 0.6 mg. atropine sulfate were given orally or subcutaneously in an attempt to control gastrointestinal effects. In only a few cases did atropine (in doses short of producing the uncomfortable side effects) prevent or relieve these symptoms. Miscellaneous symptoms followed 65% of the DFP

TABLE 4

Comparison of side effects produced by neostigmine and DFP in 6 patients with myasthenia gravis

	NEOSTIGMINE OCCURRENCE	%	DFP OCCURRENCE	%
Total doses	308		49	
Nausea	11	3.5	22	44.8
Vomiting	2	0.6	9	18
Diarrhea .	2	0.6	11	22
Headache .			9	18
Miscellaneous symptoms	19	6.1	32	65.4

doses and only 6% of the neostigmine doses. It appears that DFP is capable of producing effects other than those upon skeletal muscle or upon the peripheral portion of the autonomic nervous system. Whether the central nervous effects of DFP are due to inactivation of Ch. E. can only be surmised. It is probable that both DFP and neostigmine have actions other than those due toinactivation of Ch.E.

C. Effect of repeated administration of DFP on body functions. Repeated administration of DFP to 6 patients with myasthenia gravis, who received 11 to 210.6 mg. over periods ranging from 6 to 149 days, had no effects upon liver, kidney or hematopoietic function with the exception of H. G. who developed a severe hepatitis. The possibility of a hepatotoxic reaction to DFP was considered, but appeared to be remote for the following reasons: A total of only 11.0 mg. had been administered over a four week period (the most strongly hepatotoxic drugs do not produce liver damage even in 5 to 10 times this dosage). DFP, given over a period of several months in repeated high doses, produced no change in liver function or in liver structure in dogs, monkeys, and rats (2).

Careful studies on man have failed to reveal any liver abnormality except in this patient. The jaundice became apparent 25 days after the last injection and 50 days after the injection of 7 of the 11 mg. of DFP. Such a long latent period is not characteristic of hepatitis due to toxic drugs. The patient had received two transfusions of 500 and 300 cc. citrated blood two months previously. The latent period between these transfusions and the jaundice is quite typical of that of homologous serum jaundice. For these reasons, this reaction was classified as a virus hepatitis rather than one due to DFP.

D. Effects of single injections of DFP upon body functions. The effect of 2.0 mg. of DFP in oil (administered intramuscularly to normal adults) upon pulse,

TABLE 5

Effects of DFP (2.0 mg. in oil I. M.) and neostigmine (1.0 mg. I. M.) upon cardiovascular system

SUBJECT	DRUG	PULSE						SYSTOLIC B.P.						DIASTOLIC B.P.						"CARDIAC OUTPUT"					
		Hours																							
		0	1	2	5	9	12	0	1	2	5	9	12	0	1	2	5	9	12	0	1	2	5	9	12
B. B.	DFP	76	58	72	78	80	80	110	102	100	122	110	118	70	60	68	82	70	76	24.3	24.6	22.3	27.5	24.9	25.6
	Neostigmine	78	54	53				122	106	104				80	66	70				25.9	19.7	15.9			
W. G.	DFP	60	58	58	56	60	62	106	108	110	104	110	120	56	62	74	64	80	76	18.5	16.3	19.2	20.5	18.5	20.3
	Neostigmine	58	60	55				100	118	116				56	80	80				20.5	19.6	18.6			
J. D.	DFP	72	64	66	56	72	74	102	102	108	100	118	120	68	66	72	65	82	76	23.4	21.2	20	21.7	24.5	24.7
	Neostigmine	62	55	58				104	100	100				70	66	66				23.6	19.7	21.4			
W. S.	DFP	72	72	74	88	78	74	108	110	106	128	130	118	82	70	76	74	78	78	21.7	20.5	19.8	23	22.3	19.9
	Neostigmine	74	66	69				128	126	128				78	76	78				25.1	18.2	21.5			
P. W.	DFP	66	68	72	80	84		102	105	102	98	114		58	54	54	54	52		20.2	26.8	18.3	18.7	23.7	22.8
	Neostigmine	89	84	74				118	108	106				66	50	50				26.5	21.7	22.3			
C. B.	DFP	60	54	57	60	58	68	104	108	100	112	110	110	76	76	70	68	74	66	20.1	17.2	17.4	15.3	18.8	22.6
	Neostigmine	60	52	45				122	102	106				74	72	74				21.6	21	20.5			
J. T.	DFP	66	66	66	66	66	62	114	116	122	120	124	118	80	72	78	72	78	78	29.3	24.4	27.3	27.9	25.7	21
	Neostigmine	65	64	55				118	118	120				74	78	78				27.3	25.9	22			

blood pressure, cardiac output, vital capacity and blood sugar, is shown in tables 5, 6, and 7. There was no significant effect upon the cardiovascular system; in this respect, the action of DFP was quite similar to that of neostigmine given to the same subjects (table 5). Electrocardiographic tracings were made upon each occasion that a ballistocardiogram was taken. The former were analyzed for evidences of vagus stimulation of the heart; no consistent abnormalities were observed.

Vital capacity was measured in 5 subjects in an attempt to detect the possible occurrence of bronchoconstriction; none occurred. It is obvious that vital capacity determinations would not detect mild bronchoconstriction since muscular effort could overcome such resistance. The drug has not and should not, we believe, be given to patients with a history of asthma.

E. Simultaneous use of neostigmine and DFP. It has been reported on the basis of animal experiments employing large dosage of neostigmine and DFP (15)

TABLE 6

Effect of intramuscular injection of 2.0 mg. of DFP in oil upon the vital capacity of normal adults

	CONTROL	TIME AFTER DFP INJECTION		
		1 hr.	2 hrs.	9 hrs.
B. B.	5,480	5,570	5,440	5,660
W. G.	5,220		5,360	
J. D.	4,200	4,130		
W. S.	4,670	4,670		
P. W.	2,900	3,080		

TABLE 7

Effect of DFP upon the blood sugar of normal fasting adults

SUBJECT	BLOOD SUGAR		
	Control	1 hr. after drug	2 hrs. after drug
	*mg. per 100 cc.**	*mg. per 100 cc.**	*mg. per 100 cc.**
B. B.	90 88	87 87	86 82
W. G.	82 81	82 78	71 70
J. D.	81 79	78 75	75 74
W. S.	91 90	91 89	93 96
P. W.	88 88	93 95	95 97
C. B.	88 88	89 90	88 86
J. T.	88 88	83 81	87 88
A. C.†	85 90	83 83	82 81
M. H.†	78 75	79 78	80 80

* All determinations done in duplicate.
† Subjects received no drug.

(2) that previous administration of neostigmine prevents some or all of the actions of DFP, while the preliminary injection of DFP leads to marked augmentation

or potentiation of the effects of subsequently administered neostigmine. Since such combinations might lead to unusually marked, or unusually slight, effects therapeutically, it was necessary to determine whether the same relationship held over the range of therapeutic dosage. Two patients with myasthenia gravis who had low plasma (32 and 32%) and RBC (16 and 27%) Ch. E. levels following intensive therapy with DFP (ending 3–5 days previously) were given ascending doses (0.12, 0.24, 0.48 mg.) of neostigmine hypodermically at 4 hour intervals. No reaction followed any injection. Following this test, neostigmine and DFP were used concurrently; no increased effects were ever noted from the employment of the patient's usual dose of neostigmine at times when the blood Ch.E. levels were markedly reduced due to DFP action. No attempt was made to determine possible toxic effects from larger doses of neostigmine following the administration of DFP.

While we have no quantitative data on this point it appeared that previous administration of neostigmine to some of our patients with myasthenia gravis prevented the action of DFP (16). More precise experiments bearing on this point were performed in man by Leopold and Comroe (17) who instilled physostigmine or neostigmine into one conjunctival sac of normal individuals and, at the height of the miosis produced, then instilled DFP into the conjunctival sacs of both eyes. It was found in every case that previous administration of neostigmine or physostigmine prevented the characteristic prolonged miosis of DFP. The eyes that had received DFP alone showed marked miosis lasting for more than a week whereas in those that had neostigmine or physostigmine followed in half an hour by DFP, the miosis was of relatively short duration (one to two days). It is possible that this antagonism extends to other bodily systems.

SUMMARY AND CONCLUSIONS

1. The administration of DFP in small dosage (2–3 mg. intramuscularly) resulted in marked decrease in plasma choline esterase and slight decrease in RBC cholinesterase. The effects of DFP on the body resemble qualitatively those produced by neostigmine, but were much more prolonged. Plasma Ch.E. did not return to normal until several days or weeks after administration of a single dose of DFP; RBC Ch.E. required much longer periods of time.

2. Single injections of DFP had no consistent effect upon pulse, systolic and diastolic blood pressure, ECG, BCG, vital capacity, and blood sugar. Repeated injections produced no changes in hepatic, renal or hematopoietic functions.

3. Large or repeated doses produced symptoms which were predominantly gastrointestinal in origin (nausea, epigastric distress, vomiting, abdominal cramps, increased peristalsis and diarrhea). These are attributable to increased activity of the parasympathetic nervous system. Symptoms referable to the central nervous system were observed less frequently.

4. Administration of moderate doses of neostigmine following DFP did not produce any evident potentiation of their effects, though previous administration of neostigmine reduced or prevented the actions of DFP.

5. The rate of absorption, the ratio of oral to intramuscular dosage, and the suitability of peanut oil as a vehicle were investigated.

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STUDIES WITH TETRAZOLE DERIVATIVES[1]

I. Some Pharmacologic Properties of Aliphatic Substituted Pentamethylene Tetrazole Derivatives

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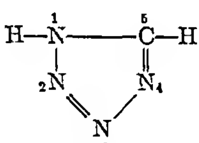
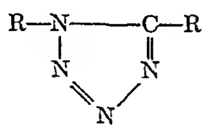
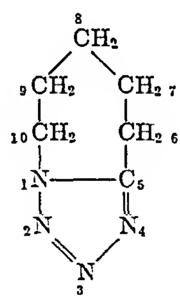
Although a relatively high degree of pharmacologic action is associated with certain tetrazole structures, descriptions of the action of only a small number of these compounds have been reported in the literature. Because of its practical usefulness as an analeptic agent, pentamethylene tetrazole (metrazol) is perhaps the best known member of this group of compounds. Other compounds of this type which have been investigated in some detail include camphor tetrazole whose actions have been described by Jackson (1), a number of methyl substituted pentamethylene and tetramethylene tetrazoles described by Issekutz, et al. (2, 3), and several polymethylene tetrazoles prepared and described by Ruzicka, et al (4). Excepting these few compounds, the literature concerning the pharmacologic action of tetrazole derivatives is particularly barren.

The tetrazole ring is unique among cyclic systems in that it offers only two points of substitution, in positions 1 and 5.



Tetrazole





A great variety of tetrazole derivatives has been described in the chemical literature. The substituents, R, may be hydrocarbon groups such as simple alkyl or aryl groups, or one substituent may be an amino group (NH_2), a sulfhydryl group (SH), a hydroxyl group (OH), a halogen or some modification of these. The pentamethylene structure shown above represents a special case of a dialkyl substituted tetrazole derivative in which the two alkyl substituents form a second cyclic system. The addition of the pentamethylene structure with ten theoretically replaceable hydrogen atoms naturally greatly enhances the possibility of substitution in the structure.

A rather wide variety of new tetrazole structures has been made available to

[1] These compounds were prepared by E. Bilhuber, Inc.

us, and it is our intention to describe in this and subsequent publications some of the pharmacologic properties of these different structures and to attempt to make some correlation between structural changes and changes in pharmacologic action. It seemed desirable for the present to classify these derivatives on the basis of the chemical structural relationships although at a later date a restatement of the results on the basis of pharmacologic action may become informative.

The first group of new tetrazole derivatives with which the present report is concerned consists of derivatives of pentamethylene tetrazole in which a variety of alkyl groups have been introduced at different points in the pentamethylene structure. Most of these compounds exhibited pharmacologic effects similar to those of the parent substance, pentamethylene tetrazole (metrazol), and were of interest primarily from the point of view of their possible usefulness as analeptic agents.

The use of analeptic agents to produce arousal in depressed states due to drugs is a common practice. The effectiveness and usefulness of an analeptic agent varies directly with its ability to stimulate respiration without producing convulsions. The greater the spread between the stimulating dose and the convulsive dose in the depressed subject the more useful the analeptic agent. Pentamethylene tetrazole (metrazol) which has been widely used as a respiratory stimulant is very prompt in its action and its effect is of relatively short duration and not cumulative. It has been used as the standard for comparison in this investigation.

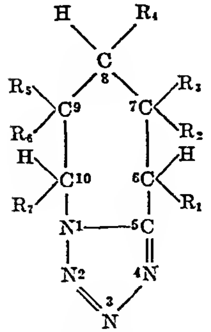
Issekutz and co-workers (2) have reported that the introduction of a methyl group into the pentamethylene tetrazole structure (Compounds TT-1 and TT-6 in tables 1 and 2) enhances the activity of the parent structure. We have been able to confirm their results. In view of this fact, it appeared desirable to extend the series of alkyl substituted pentamethylene tetrazole derivatives to learn whether the maximum enhancement of activity had been attained and to study in more detail the analeptic action of the various compounds. The structures of the alkylated pentamethylene tetrazoles were varied systematically and an effort was made to correlate these structural changes with changes in the pharmacological action. Both the convulsant and analeptic actions of twenty-five derivatives of pentamethylene tetrazole were determined and the results are summarized in table 2. The chemical structures of all the compounds are shown in table 1.

EXPERIMENTAL. The stimulatory action of these compounds upon the central nervous system was studied by injection of graded doses into albino rats of approximately equal weights. The water-soluble compounds were administered intraperitoneally, while water-insoluble compounds were dissolved in dibutyl succinate and injected intramuscularly. The toxic effect of dibutyl succinate when given intraperitoneally (5) necessitated administration of these solutions by the intramuscular route. For comparative purposes pentamethylene tetrazole was administered both in water and in bibutyl succinate solution. The dose in normal rats did not exceed 1000 mgm./kgm. Convulsive and lethal effects within this limit were recorded.

Arousal action was studied on rats, rabbits and dogs under pentobarbital sodium anesthesia. An anesthetic dose of pentobarbital sodium (35 mgm./kgm.) was administered intraperitoneally in rats and intravenously in rabbits and dogs. After deep anesthesia had

TABLE 1

Structural relationships



Substituted pentamethylene tetrazole

COMPOUND NO	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Metrazol	H	H	H	H	H	H	H
TT-1	CH_3	H	H	H	H	H	H
TT-7	H	CH_3	H	H	H	H	H
TT-6	H	H	H	CH_3	H	H	H
TT-011	H	H	H	iso-C_3H_7	H	H	H
TT-012	H	H	H	sec. C_4H_9	H	H	H
TT-94	H	H	H	tert. C_4H_9	H	H	H
TT-90	H	H	H	tert. C_5H_{11}	H	H	H
TT-87	H	H	H	C_6H_{11}	H	H	H
TT-15	H	CH_3	H	H	CH_3	H	H
TT-8	H	CH_3	$COOCH_3$	H	CH_3	H	H
TT-54	H	CH_3	COOH	H	CH_3	H	H
TT-11	H	CH_3	H	H	C_2H_5	H	H
TT-63	H	CH_3	$COOCH_3$	H	C_2H_5	H	H
TT-64	H	CH_3	COOH	H	C_2H_5	H	H
TT-13	H	CH_3	H	H	n-C_3H_7	H	H
TT-52	H	CH_3	H	H	iso-C_3H_7	H	H
TT-55	H	CH_3	$COOCH_3$	H	iso-C_3H_7	H	H
TT-57	H	CH_3	COOH	H	iso-C_3H_7	H	H
TT-22	H	CH_3	H	H	H	H	iso-C_3H_7
TT-4	H	CH_3	H	H	CH_3	CH_3	H
TT-53	H	CH_3	COOH	H	CH_3	CH_3	H
TT-56	H	CH_3	$CON\langle{}^{C_2H_5}_{C_2H_5}$	H	CH_3	CH_3	H
TT-40 (a)	H	H	H	H	H	H	H
TT-41 (b)	H	H	H	H	H	H	H
TT-0102 (c)	H	H	H	tert. C_4H_9	H	H	H

(a) Metrazol methyl benzene sulfonate (quaternary salt).
(b) Metrazol methiodide (quarternary salt).
(c) 8-tert. Butyl pentamethylene tetrazole methyl benzene sulfonate (quaternary salt).

1. The synthesis and chemical properties of these compounds will be described elsewhere

2 It should be pointed out that the methods employed in the synthesis of these compounds could in certain cases lead to isomeric products. Although each compound described in table 1 is a pure, homogenous product, the possibility exists in certain cases that the compounds have the isomeric structure which would be represented by transposition of the substituent groups in positions 7 and 9, and 6 and 10.

TABLE 2

Convulsant, lethal and arousal doses

COMPOUND NO.	ANIMAL	SOLVENT USED	MINIMUM CONVULSIVE DOSE	MINIMUM LETHAL DOSE	AROUSAL FOLLOWING PENTOBARBITAL ANESTHESIA, MINIMAL REQUIRED	ROUTE OF ADMINISTRATION
			mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
Metrazol	Rat	Water	25	50	55–60	I.P.
Metrazol	Rat	Succinate	35	60	70–80	I.M.
Metrazol	Rabbit	Water			45–50	I.V.
Metrazol	Rabbit	Succinate			75	I.M.
TT-1	Rat	Water	30	35	100	I.P.
TT-1	Rabbit	Water			40	I.V.
TT-7	Rat	Water	10	25	50	I.P.
TT-7	Rabbit	Water			15‡	I.V.
TT-6	Rat	Water	4	8	5–10 (I.P.)	I.M.
TT-6	Rabbit	Water			5–10	I.V.
TT-6	Dog	Water			10	I.V.
TT-011	Rat	Succinate	3	8	15	I.M.
TT-012	Rat	Succinate	750	900		I.M.
TT-94	Rat	Succinate	3	5	20	I.M.
TT-90	Rat	Succinate	150	200		I.M.
TT-87	Rat	Succinate	*, **			I.M.
TT-15	Rat	Succinate††	†			I.M.
TT-8	Rat	Succinate	600			I.M.
TT-54	Rat	Water	*			I.P.
TT-11	Rat	Succinate	*			I.M.
TT-63	Rat	Succinate	†			I.M.
TT-64	Rat	Water	*			I.P.
TT-13	Rat	Succinate	*			I.M.
TT-52	Rat	Succinate	300	500	600–700	I.M.
TT-55	Rat	Succinate	*			I.M.
TT-57	Rat	Water	*			I.P.
TT-22	Rat	Succinate	500			I.M.
TT-4	Rabbit	Succinate			150 (slight)	I.M.
TT-53	Rat	Water	†			I.P.
TT-56	Rat	Succinate	†			I.M.
TT-40	Rat	Water	†			I.P.
TT-41	Rat	Water	†			I.P.
TT-0102	Rat	Water	300§	500		I.P.

* Negative up to 1,000 mgm./kgm.
† Negative up to 500 mgm./kgm.
‡ 20 to 30 mgm./kgm. doses required for the animal to assume a sitting position.
§ Mixed depression and convulsions.
** Quieting and sedation.
†† Succinate + 10% ethanol.

developed, graded doses of the experimental compounds were administered and the arousal effects noted. The criterion of arousal in rats was their ability to assume a crawling position, in rabbits and dogs, the ability to sit up and raise the head. The minimal doses required to fulfill these criteria were determined.

Certain compounds which showed considerable stimulatory action in low doses and

appeared to have fairly low toxicity were further studied for their ability to increase respiration depressed by morphine or pentobarbital sodium. Changes in minute volume and respiratory rate as compared with the normal were recorded after administration of pentobarbital sodium (35 mgm./kgm.) or morphine (3 mgm./kgm.) and subsequent administration of the test compound, orally in propylene glycol solution or intravenously in aqueous solution.

RESULTS. A summary of the convulsive, arousal and toxic doses of the new compounds is presented in table 2.

The effectiveness as respiratory stimulants of several compounds which exhibited favorable action as arousal agents can be judged from the data in table

TABLE 3

Effect of oral administration of tetrazole derivatives on respiration depressed with pentobarbital sodium

TIME AFTER ADMINISTRATION OF STIMULANT (MINUTES)	RABBITS, 35 MGM./KGM. PENTOBARBITAL SODIUM						DOGS	
	Metrazol, 100 mgm./kgm.		TT-94, 50 mgm./kgm.		TT-52, 100 mgm./kgm.		TT-94, 50 mgm./kgm.	
	Resp vol.	Minute rate	Resp. vol.	Minute rate	Resp. vol	Minute rate	Resp vol.	Minute rate
	cc/min		cc./min.		cc/min.			
(Normal)	2,400	124	1,600	120	1,700	130		
Depressed	950	46	700	44	500	26		24
5	1,100	52	1,050	78	400	12		21
10	900	48	1,400	72	700	26		47
15	850	46			600	32		
20	1,100	58	1,000	60	600	32		48
25	1,150	68			1,000	46		
30	1,200	76	1,000	60	800	38		24
35	1,450	78			900	42		
40								
45	1,700	104			900	44		
50	1,750	136						
55	1,600	132			900	44		
60	1,700	124	1,050	60				24

3. The effect of compounds TT-6, TT-94 and TT-52 on the respiratory rate and minute volume of rabbits or dogs under pentobarbital sodium or morphine depression was compared with metrazol as a standard. The minute volume was recorded by a calibrated spirometer using a two way respiratory valve. Data recorded in the table are typical examples. Oral administration of 100 mgm./kgm. of metrazol as a 5% solution in propylene glycol to rabbits anesthetized with pentobarbital sodium caused a slow progressive return to normal of both respiratory rate and volume with complete arousal. Compound TT-52 (100 mgm./kgm. as 5% propylene glycol solution orally administered) gave a considerably slower progressive rise in rate and volume for 30 minutes followed by a slight recession from the maximum level attained. The only arousal effect noted was the return of the wink at peak effect. Oral administration of 50

TABLE 2

Convulsant, lethal and arousal doses

COMPOUND NO.	ANIMAL	SOLVENT USED	MINIMUM CONVULSIVE DOSE	MINIMUM LETHAL DOSE	AROUSAL FOLLOWING PENTOBARBITAL ANESTHESIA, MINIMAL REQUIRED	ROUTE OF ADMINISTRATION
			mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
Metrazol	Rat	Water	25	50	55–60	I.P.
Metrazol	Rat	Succinate	35	60	70–80	I.M.
Metrazol	Rabbit	Water			45–50	I.V.
Metrazol	Rabbit	Succinate			75	I.M.
TT-1	Rat	Water	30	35	100	I.P.
TT-1	Rabbit	Water			40	I.V.
TT-7	Rat	Water	10	25	50	I.P.
TT-7	Rabbit	Water			15‡	I.V.
TT-6	Rat	Water	4	8	5–10 (I.P.)	I.M.
TT-6	Rabbit	Water			5–10	I.V.
TT-6	Dog	Water			10	I.V.
TT-011	Rat	Succinate	3	8	15	I.M.
TT-012	Rat	Succinate	750	900		I.M.
TT-94	Rat	Succinate	3	5	20	I.M.
TT-90	Rat	Succinate	150	200		I.M.
TT-87	Rat	Succinate	*, **			I.M.
TT-15	Rat	Succinate††	†			I.M.
TT-8	Rat	Succinate	600			I.M.
TT-54	Rat	Water	*			I.P.
TT-11	Rat	Succinate	*			I.M.
TT-63	Rat	Succinate	†			I.M.
TT-64	Rat	Water	*			I.P.
TT-13	Rat	Succinate	*			I.M.
TT-52	Rat	Succinate	300	500	600–700	I.M.
TT-55	Rat	Succinate	*			I.M.
TT-57	Rat	Water	*			I.P.
TT-22	Rat	Succinate	500			I.M.
TT-4	Rabbit	Succinate			150 (slight)	I.M.
TT-53	Rat	Water	†			I.P.
TT-56	Rat	Succinate	†			I.M.
TT-40	Rat	Water	†			I.P.
TT-41	Rat	Water	†			I.P.
TT-0102	Rat	Water	300§	500		I.P.

* Negative up to 1,000 mgm./kgm.
† Negative up to 500 mgm./kgm.
‡ 20 to 30 mgm./kgm. doses required for the animal to assume a sitting position.
§ Mixed depression and convulsions.
** Quieting and sedation.
†† Succinate + 10% ethanol.

developed, graded doses of the experimental compounds were administered and the arousal effects noted. The criterion of arousal in rats was their ability to assume a crawling position, in rabbits and dogs, the ability to sit up and raise the head. The minimal doses required to fulfill these criteria were determined.

Certain compounds which showed considerable stimulatory action in low doses and

appeared to have fairly low toxicity were further studied for their ability to increase respiration depressed by morphine or pentobarbital sodium. Changes in minute volume and respiratory rate as compared with the normal were recorded after administration of pentobarbital sodium (35 mgm /kgm) or morphine (3 mgm./kgm.) and subsequent administration of the test compound, orally in propylene glycol solution or intravenously in aqueous solution.

RESULTS. A summary of the convulsive, arousal and toxic doses of the new compounds is presented in table 2.

The effectiveness as respiratory stimulants of several compounds which exhibited favorable action as arousal agents can be judged from the data in table

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	Metrazol, 100 mgm / kgm		TT-94, 50 mgm /kgm		TT-52, 100 mgm / kgm		TT-94, 50 mgm / kgm	
	Resp vol	Minute rate	Resp vol	Minute rate	Resp vol	Minute rate	Resp vol	Minute rate
	cc /min		cc /min		cc /min			
(Normal)	2,400	124	1,600	120	1,700	130		
Depressed	950	46	700	44	500	26		24
5	1,100	52	1,050	78	400	12		21
10	900	48	1,400	72	700	26		47
15	850	46			600	32		
20	1,100	58	1,000	60	600	32		48
25	1,150	68			1,000	46		
30	1,200	76	1,000	60	800	38		24
35	1,450	78			900	42		
40								
45	1,700	104			900	44		
50	1,750	136						
55	1,600	132			900	44		
60	1,700	124	1,050	60				24

3. The effect of compounds TT-6, TT-94 and TT-52 on the respiratory rate and minute volume of rabbits or dogs under pentobarbital sodium or morphine depression was compared with metrazol as a standard. The minute volume was recorded by a calibrated spirometer using a two way respiratory valve. Data recorded in the table are typical examples. Oral administration of 100 mgm./kgm. of metrazol as a 5% solution in propylene glycol to rabbits anesthetized with pentobarbital sodium caused a slow progressive return to normal of both respiratory rate and volume with complete arousal. Compound TT-52 (100 mgm /kgm. as 5% propylene glycol solution orally administered) gave a considerably slower progressive rise in rate and volume for 30 minutes followed by a slight recession from the maximum level attained. The only arousal effect noted was the return of the wink at peak effect. Oral administration of 50

mgm./kgm. of compound TT-94 as a 5% solution in propylene glycol to rabbits under pentobarbital anesthesia caused the respiratory volume to rise rapidly to the normal level within 10 minutes but the respiratory rate failed to show a comparable increase. After this peak of action, the rate and volume dropped considerably so that the beneficial effects were dissipated at the end of an hour. Increasing the dose of compound TT-94 to 75 mgm./kgm. produced convulsions after 30 minutes. Similar effects were observed in a dog when compound TT-94 was given orally (50 mgm./kgm. in 5% propylene glycol solution) after induction of pentobarbital sodium anesthesia. After 20 minutes there was a marked increase in respiratory rate; and muscular movements, vomiting and defecation were noted. After induction of pentobarbital sodium anesthesia in a dog the intramuscular injection of 10 mgm./kgm. of compound TT-6 caused a slight

TABLE 4

Effect of compound TT-6 on respiration of rabbits depressed with morphine

	TIME AFTER ADMINISTRATION OF DRUG	RESPIRATORY RATE	RESPIRATORY VOLUME
	minutes		*cc./min.*
Normal		88	1,350
Morphine sulfate (3 mgm./kgm.)	5	56	1,700
	10	48	1,050
	15	42	850
	20	42	750
TT-6 (0.5 mgm./kgm.) I.V.	1	110	3,200
	5	88	3,100
	10	78	2,950
	15	76	2,300
	20	66	1,850
	40	60	1,475

increase in respiratory rate and return of the wink reflex. A second dose of 10 mgm./kgm. of compound TT-6 given 20 minutes later caused a further increase in respiratory rate, but also produced convulsions which were controlled with pentobarbital sodium.

In rabbits with respiration depressed by 3 mgm./kgm. of morphine sulfate intravenously, the administration of compound TT-6 (0.2 mgm./kgm. by vein) (see table 4) caused an immediate return of the respiratory rate and volume to somewhat over the normal level. After 5 minutes, however, both rate and volume had dropped to the low morphine level. These results are comparable to the effects produced by a 2 mgm./kgm. intravenous dose of metrazol. A second dose of 0.5 mgm./kgm. of compound TT-6 in the same animal caused slight convulsions for 30 seconds. The respiratory volume increased to twice the normal level, but had returned to the normal level after 20 minutes while the rate failed to rise, and after the 20 minute period was still considerably below the normal level.

DISCUSSION. The results outlined and summarized above indicate that certain changes in the pentamethylene tetrazole structure may greatly enhance its stimulatory effects on the central nervous system. Although a great increase in the potency could be effected by structural changes, these were generally accompanied by a decrease in the efficiency of the compounds as analeptics. Rather than widening the gap between convulsive dose and analeptic dose, the reverse has taken place. In no case does the general character of the action of these compounds show an improvement over that of the parent structure and in addition, the valuable adjunct of water solubility has been sacrificed in most cases.

The following changes in pharmacologic properties appear to be effected by alterations in the molecule: (a) the introduction of a single methyl group into the pentamethylene tetrazole structure caused increasing enhancement of the activity as the group is moved successively from position 6 and 7 to position 8. The solubility of the parent structure in water is greatly decreased by the introduction of a single methyl group. (b) Increasing the size of a single alkyl group in position 8 of the pentamethylene structure makes the resulting compound practically insoluble in water and has a mixed effect upon the pharmacologic action. Thus if the size of the substituent in position 8 is increased by the replacement of the hydrogen atoms of the substituent methyl group in compound TT-6 by additional methyl groups, the activity is further enhanced. For instance the 8-isopropyl and 8-tert. butyl pentamethylene tetrazoles are more active than the 8-methyl compound. On the other hand, simply increasing the size of the substituent in position 8 as by the introduction of a secondary butyl or tertiary amyl group causes a very marked decrease in activity, to the extent that 8-cyclohexyl pentamethylene tetrazole is actually a depressant rather than a stimulant. (c) Introduction of a second alkyl group causes practically complete disappearance of the water solubility as well as profound reduction in the activity. Two exceptions should be noted. In compounds with a methyl group in position 7 and various alkyl groups in position 9, no activity was noted as the size of the 9-alkyl group was increased except in the case of the 9-isopropyl compound, where a moderate degree of analeptic and convulsant action was encountered (compound TT-52). When the isopropyl group was shifted to position 10 (compound TT-22) some reduction of the activity was observed The effect of the isopropyl group in bestowing analeptic and convulsant activity upon these compounds whether as a single substituent or as a second substituent is noteworthy. The introduction of three methyl groups as substituents (compound TT-4) had only a slight effect; a moderate improvement in analeptic activity in rabbits over the dimethyl derivative (compound TT-15) was noted. (d) The introduction of a carboxyl group, although it bestowed water solubility upon the compounds as sodium or potassium salts only caused further reduction of convulsant and analeptic activity. Even the methyl esters of these acids failed to exhibit much activity although water solubility was sacrificed (e) The formation of quaternary salts with methyl benzene sulfonate or with methyl iodide, although it bestowed water solubility on the compounds, markedly decreased their activity. The parent pentamethylene tetrazole (metrazol) became completely inactive (compounds TT-40 and TT-41) while the more

active 8-tertiary butyl pentamenthylene tetrazole (compound TT-94) lost much of its activity when converted into the quaternary salt (compound TT-0102). This loss in activity may be due to an alteration in the relative solubilities of these compounds in water and fat solvents on conversion to quaternary salts.

SUMMARY

A few generalizations can be made from the data in Table 2. The introduction of a single substituent, R_4, into the pentamethylene tetrazole structure will enhance the analeptic and convulsant activity of the parent substance only when the group is small, or closely packed as in the case of methyl, isopropyl or tert.butyl. Substitution of alkyl groups in positions other than R_4, di- and trisubstitutions, or the formation of quaternary salts decreased the activity of the compounds studied. The isopropyl group appeared to be unique in the latter groups, since in any given case it was generally associated with the most active compounds. None of the new compounds appears to have advantages over the parent compound when toxicity and solubility are considered.

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STUDIES WITH TETRAZOLE DERIVATIVES

II. Some Pharmacologic Properties of 1,5-Disubstituted Tetrazoles[1]

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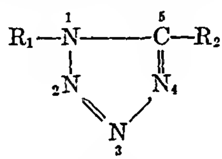
In the first paper of this series (1), a group of pentamethylene tetrazole derivatives was studied in an effort to determine their convulsant and analeptic actions as compared with pentamethylene tetrazole. The second group of tetrazole derivatives, with which the present report is concerned, consists of derivatives of tetrazole in which a variety of alkyl groups or the phenyl group has been introduced at the only two possible points of substitution, in positions 1 and 5.



The structures of the substituted tetrazoles were varied systematically and an attempt was made to correlate the changes in chemical structure and pharmacological activity. The schema for the systematic alteration of substituent groups is presented in table 1. Only five of these compounds have been previously described in the chemical literature, namely: TT-0148, TT-9, TT-21, TT-20 and TT-19 (2, 3, 4, 5, 6). No record of a description of the pharmacologic properties of any of them appears to exist. Both the stimulatory and analeptic actions of twenty-five derivatives of tetrazole were determined and the results are summarized in table 2.

Experimental. The techniques used in these studies were identical with those reported in the first paper of this series.

Results. The results of a determination of the convulsant, arousal and toxic doses of these compounds are summarized in table 2.

In several cases further studies were carried out. The parent compound, tetrazole (TT-0148), when administered to rabbits under pentobarbital anesthesia in doses of 50 and 100 mgm./kgm. intraperitoneally failed to cause any arousal. This result was not unexpected in view of the absence of stimulatory action in rats.

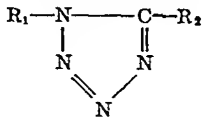
The effect of two compounds on respiration was studied in some detail. The oral administration of TT-79, 50 mgm./kgm. as a 5% solution in propylene glycol to rabbits under pentobarbital anesthesia caused a progressive rise in respiratory rate and volume for 30 minutes at which time the animals were sitting up in a normal manner. Increasing the dose to 100 mgm./kgm. caused

[1] These compounds were prepared by E. Bilhuber, Inc.

no greater action. At either dosage level the recovery of respiratory rate and volume was complete. Similarly an oral dose of 50 mgm./kgm. of TT-18 in 5% propylene glycol solution caused a rapid rise in respiratory volume in a rabbit under pentobarbital anesthesia, recovery being complete in about 10 minutes. The respiratory rate, however, did not increase in the same degree. At the end

TABLE 1

Structural relationships



CODE NO.	R_1	R_2
TT-0148	H	H
TT-9	CH_3	CH_3
TT-020	C_4H_9 (iso)	CH_3
TT-10	C_4H_9 (iso)	C_4H_9 (iso)
TT-21	C_6H_5	CH_3
TT-27	C_6H_5	C_2H_5
TT-47	C_6H_5	C_4H_9 (iso)
TT-44	C_6H_5	C_6H_{11} (cyclohexyl)
TT-20	C_6H_5	C_6H_5
TT-19	CH_3	C_6H_5
TT-49	C_2H_5	C_6H_5
TT-45	C_4H_9 (iso)	C_6H_5
TT-48	C_6H_{11}	C_6H_5
TT-79	C_6H_{11}	CH_3
TT-014	C_6H_{11}	CH_2OH
TT-014 Ac	C_6H_{11}	CH_2OCOCH_3
TT-18	C_6H_{11}	C_2H_5
TT-067	C_6H_{11}	C_3H_7 (n)
TT-071	C_6H_{11}	C_3H_7 (iso)
TT-073	C_6H_{11}	C_4H_9 (n)
TT-17	C_6H_{11}	C_4H_9 (iso)
TT-5	C_6H_{11}	C_6H_{11}
TT-24	CH_3	C_6H_{11}
TT-14	C_2H_5	C_6H_{11}
TT-51	C_4H_9 (iso)	C_6H_{11}
TT-09	C_5H_9 (cyclopentyl)	CH_3

of an hour these beneficial effects seemed to dissipate. Doubling the dose did not appear to be effective in prolonging the action of the compound. Data showing the effect of these two compounds are given in table 3.

DISCUSSION. Certain compounds in this group appear to exhibit central nervous stimulation and analeptic action which would make them appear worthy of more extensive investigation. In spite of their insolubility in water their actions appear promptly whether administered intramuscularly or orally. They compare favorably with metrazol in speed of action, potency and smoothness of

TABLE 2

Convulsant, lethal and arousal doses

COMPOUND NO.*	ANIMAL	MINIMUM CONVULSIVE DOSE	MINIMUM LETHAL DOSE	AROUSAL FOLLOWING PENTOBARBITAL ANESTHESIA, MINIMAL REQUIRED	COMMENT
		mgm /kgm	*mgm /kgm*	*mgm /kgm.*	
TT-0148	Rat	No action			Maximum dose used: 1000 mgm./kgm.
TT-9	Rat	No action			Slight sedation above 750 mgm./kgm.
TT-020	Rat	40	200	350	
TT-10	Rat	No action			
TT-21	Rabbit			100	125 mgm./kgm. fatal
TT-21	Rat	750			Early sedation followed by latent restlessness, excitement and muscle tremors
TT-27	Rat	400	700		Doses less than 400 mgm./kgm. slightly sedative. Lethal effect latent
TT-47	Rat	No action			Doses greater than 600 mg./kgm. slightly sedative
TT-44	Rat	No action			Maximum dose used: 750 mgm./kgm.
TT-20					Not tested, insoluble in HOH and succinate
TT-19	Rat	No action			Doses above 300 mgm./kgm. slightly sedative
TT-49	Rat	No action			Doses of 750 mgm./kgm. sedative but responds to stimuli
TT-45	Rat	No action			Maximum dose used: 800 mgm./kgm.
TT-48	Rat	No action			Maximum dose used: 750 mgm./kgm.
TT-79	Rat	6	50		
TT-79	Rat			75	
TT-79	Rabbit			50	
TT-014	Rat	No action			Maximum dose used: 500 mgm /kgm.
TT-014 Ac	Rat	No action			Maximum dose used: 1,000 mgm /kgm
TT-18	Rat	200	350	500	
TT-067	Rat	No action			Maximum dose used: 1,000 mgm /kgm.
TT-071	Rat	No action			Maximum dose used: 1,000 mgm./kgm.
TT-073	Rat	No action			Maximum dose used: 1,000 mgm./kgm.
TT-17	Rat	No action			Maximum dose used: 500 mgm./kgm.

TABLE 2—*Continued*

COMPOUND NO.*	ANIMAL	MINIMUM CONVULSIVE DOSE	MINIMUM LETHAL DOSE	AROUSAL FOLLOWING PENTOBARBITAL ANESTHESIA, MINIMAL REQUIRED	COMMENT
		mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
TT-5	Rat	No action			Maximum dose used: 500 mgm./kgm.
TT-24	Rat	350	600		
TT-14	Rat	No action			Doses of 500 mgm./kgm. and up: slight sedation and ataxia
TT-51	Rat	No action			Doses of 200 mgm./kgm. and up: slight sedation
TT-09	Rat	150	400	1,000	Lethal effect latent

* Compounds TT-0148 and TT-9 given in aqueous solution; all others in dibutyl succinate solution.†

† Aqueous solutions given intraperitoneally, dibutyl succinate solutions given intramuscularly.

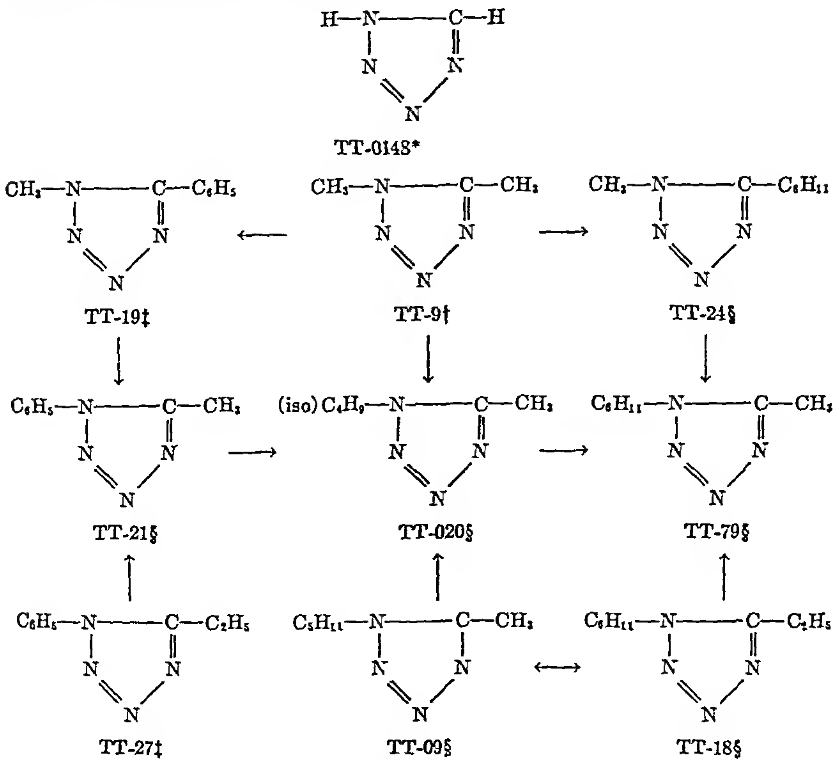
TABLE 3

TIME AFTER ADMINISTRATION OF STIMULANT (MINUTES)	RABBITS							
	TT-18, 50 mgm./kgm.		TT-18, 100 mgm./kgm.		TT-79, 50 mgm./kgm.		TT-79, 100 mgm./kgm.	
	Resp. vol.	Minute rate	Resp. vol.	Minute rate	Resp. vol.	Minute rate	Resp. vol.	Minute rate
	cc./min.		*cc./min.*		*cc./min.*		*cc./min.*	
(Normal)	2,200	144	1,800	158	1,550	148	1,800	154
			1,600	128				
Anesthesia administered								
1 (min. after)	1,500	52	750	52	600	42	950	44
4 (min. after)	800	40	650	40	550	38		
Stimulant administered								
5	1,600	72	1,350	62	750	48	1,250	60
10	1,600	94	1,000	58	1,050	88	1,450	84
15	1,600	74	800	54	1,000	88	1,500	70
20	1,300	64					1,300	70
25					1,150	96		
30			900	50				
35	1,200	76						
40			1,100	72	1,350	108	1,400	122
45								
50	900	56						
55								
60			800	64	1,600	156		

arousal, and generally appear to be longer acting. Further investigations of these compounds will form the subject of a later report.

Inspection of the data recorded in table 2 indicates certain relationships

between changes in chemical structure and pharmacologic action which can be represented schematically below:



Arrows indicate direction of increased stimulatory action
* Inactive
† Sedative
‡ Sedation followed by stimulation as dose is increased
§ Stimulation

In the schematic representation above it should be observed that the parent compound, tetrazole (TT-0148), showed no central nervous action, either stimulatory or depressant. Introduction of methyl groups into the 1 and 5 positions endowed the resulting compound, TT-9, with depressant properties. Further modification of the structure by replacing the methyl group in position 1 with a phenyl group (TT-21) led to a compound having mild stimulatory action, while transposition of the phenyl and methyl groups ((TT-19) resulted in a weakly depressant compound. On the other hand, if either of the methyl groups of TT-9 was replaced by a cyclohexyl group, a relatively potent stimulant resulted (TT-79, 1-cyclohexyl-5-methyl tetrazol and its position isomer, TT-24). The former exhibited stimulatory action of a much higher order than the latter. Likewise replacement of the methyl group in position 1 of TT-9 by an iso-butyl

group (TT-020), or a cyclopentyl group (TT-09) bestowed profound stimulatory action on the resulting compounds. Thus far it would appear that increasing the size of the substituent in position 1 of the tetrazole ring markedly increased the stimulatory activity of the resulting compound.

If in a given case, for instance TT-79, the size of the substituent in position 5 was increased, a decrease in stimulatory action followed as in the case of TT-18 which differed from TT-79 by the replacement of the methyl group in the latter with an ethyl group. Further increase in the size of the substituent either by lengthening or branching the chain (TT-067, TT-071, TT-073, TT-17) or by introducing a second cyclic structure ((TT-5, TT-48) caused complete loss of activity. Similar trends appear when data for other related groups in table 2 are considered. Thus it would appear that the conditions leading to optimum stimulatory action are the presence of a relatively large group, cyclic or open chain, in position 1 and a small group, methyl or ethyl, in position 5. Reversal of the position of the substituents leads to a marked decrease of stimulatory activity and even to reversal of action from stimulatory to depressant in weakly active compounds.

It is noteworthy that the replacement of the methyl group in the highly active TT-79 by a hydroxy-methyl group as in TT-014 completely destroyed the activity of the resulting product. Covering the alcoholic hydroxyl group by esterification with acetic acid (TT-014 Ac) failed to restore the stimulatory action.

The conditions leading to enhancement of stimulatory activity are in certain respects reminiscent of the conditions leading to maximum depressant action in the barbituric acid group. There the optimum effect is observed within narrow limits of the combined size of the two substituents on the methylene carbon. In the tetrazole series, the combined size of the substituents in positions 1 and 5 seems to reach a limiting value for optimum stimulatory activity. In general the requirements seem to be a moderately large group in position 1 and a small group in position 5. Groups of equal size, even when their combined size is near the optimum value, do not appear to produce the optimum effect. The approximate equivalence of the isobutyl, cyclopentyl and cyclohexyl groups in position 1 is of interest.

SUMMARY

The action of a group of 1,5-disubstituted tetrazole derivatives on the central nervous system has been studied. Both stimulatory and depressant effects have been observed. Certain relationships between chemical structure and pharmacologic action have become apparent. The optimum structural factors for maximum stimulatory action appear to be the presence of a relatively large saturated cyclic or open chain aliphatic group in position 1 and a small group, preferably methyl, in the 5 position of the tetrazole ring system. Several compounds in the group, namely 1-cyclohexyl-5-methyl tetrazole (TT-79), 1-cyclohexyl-5-ethyl tetrazole (TT-18), 1-cyclopentyl-5-methyl tetrazole (TT-09) and 1-isobutyl-5-methyl tetrazole (TT-020) are outstanding in their stimulatory effects and deserve further investigation.

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group (TT-020), or a cyclopentyl group (TT-09) bestowed profound stimulatory action on the resulting compounds. Thus far it would appear that increasing the size of the substituent in position 1 of the tetrazole ring markedly increased the stimulatory activity of the resulting compound.

If in a given case, for instance TT-79, the size of the substituent in position 5 was increased, a decrease in stimulatory action followed as in the case of TT-18 which differed from TT-79 by the replacement of the methyl group in the latter with an ethyl group. Further increase in the size of the substituent either by lengthening or branching the chain (TT-067, TT-071, TT-073, TT-17) or by introducing a second cyclic structure ((TT-5, TT-48) caused complete loss of activity. Similar trends appear when data for other related groups in table 2 are considered. Thus it would appear that the conditions leading to optimum stimulatory action are the presence of a relatively large group, cyclic or open chain, in position 1 and a small group, methyl or ethyl, in position 5. Reversal of the position of the substituents leads to a marked decrease of stimulatory activity and even to reversal of action from stimulatory to depressant in weakly active compounds.

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THE SPECIFICITY OF SEX HORMONES ON THE TISSUE ALDEHYDE SHIFT IN THE RAT KIDNEY AND OF FUCHSIN SULFUROUS ACID REAGENT ON ALDEHYDES[1]

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A cyclic change in the histochemical structure of the rat kidney synchronized with the estrous cycle has been recently described (1). As determined by the fuchsin sulfurous acid reaction, the tissue aldehydes in the intercortico-medullary (ICM) zone of the female rat kidney increase in the estrous phase of the cycle and tend to decrease or disappear in the diestrous or resting phase of the cycle and during pregnancy. It was observed that administration of testosterone exerts an aldehyde depositing influence and that estrogenic substances and progesterone effect an aldehyde depletion in the kidney (2).

With this phenomenon established, demonstrating for the first time a decided histochemical difference between female and male rat kidneys, a question presented itself regarding the specificity of the sex hormones on the aldehyde shift and the specificity of the fuchsin sulfurous acid reagent for the detection of aldehydes. The present investigation is concerned with a study of these problems.

Experimental. Frozen sections 50 micra in thickness were cut from the fresh unfixed kidneys of the various rats used. The sections were treated with a 1% solution of $HgCl_2$ for five minutes to liberate the bound aldehydes, washed in physiological saline and placed in a solution of fuchsin sulfurous acid (FSA). Free aldehydes present in the sections attained a maximum purple color after fifteen minutes contact.

All animals were maintained on a normal mixed diet enriched with vitamins. Steroids for injection were suspended in peanut oil and injected intramuscularly, with the exception of methyl testosterone which was administered orally. Diethylstilbestrol was fed in 1 mgm. doses dissolved in peanut oil and was given to the animals for at least seven successive days. This procedure assures a complete disappearance of the tissue aldehydes in the ICM zone of the kidney. The actual tests were begun at least one week after the last feeding of diethylstilbestrol to eliminate any possible interference by this estrogen.

As in previous publications (1, 2), a system of − and + is once more utilized to designate the degree of stainability of the kidney ICM zone and so to indicate the absence or presence of tissue aldehydes in this area. A − − ICM zone, a pale band in the stained section, is lacking in stainable tissue aldehydes, while a + + zone is deeply stained and indicates the presence of a large amount of these substances. Varying combinations of − and + are used to designate the intermediate degrees of stainability of the ICM zone.

Results and discussion. *Specificity of sex hormones on the tissue aldehyde shift in the rat kidney.* We were greatly interested in knowing if the described aldehyde shift could also be obtained by administration of androsterone deriva-

[1] Delivered in part before the Section of Biological Chemistry of the American Chemical Society at its semiannual meeting, Atlantic City, New Jersey, April, 1946.

tives. Five derivatives with greater or lesser androgenic activity were administered to ovariectomized rats, the kidneys of which had been previously depleted of aldehydes by feeding diethylstilbestrol, 1 mgm. in 0.1 cc. peanut oil, for seven successive days.

Table 1 shows the effects of these steroids on the kidneys. Each androsterone derivative, testosterone propionate, methyl testosterone, methyl androstendiol, dehydroandrosterone acetate, androstendiol and androstendione, was found to exhibit a tendency, in varying degrees, toward depositing tissue aldehydes in the ICM zones of the kidneys. Pregnenolone did not augment the stainability of

TABLE 1

The effect of certain androgenic steroids on the intercortico-medullary zones of castrated rats pretreated with diethylstilbestrol

NO OF RATS	ICM ZONE, L KIDNEY*	SUBSTANCE GIVEN	OBSERVATION PERIOD	DAILY DOSAGE	ADMINISTRATION	ICM ZONE, R, KIDNEY
			days			
4	−−	Peanut oil	11	0 3 cc.	10 I M.	−−
2	−−	Testosterone propionate	11	3.0 mg.	4 I M.	++
5	−−	Testosterone propionate	11	3 0 mg.	3 I.M.	++
2	−−	Testosterone propionate	20	3.0 mg.	3 I M.	++
2	−−	Testosterone propionate	11	3 0 mg.	1 I.M	++
2	−−	Methyl testosterone	11	3.0 mg.	10 oral	(−)(+)
2	−−	Methyl testosterone	10	9.0 mg.	9 oral	++
2	−−	Methyl androstendiol	10	9.0 mg.	8 I M	++
2	−−	Dehydroandrosterone acetate	10	9.0 mg.	8 I M.	(+)+
2	−−	Androstendiol	11	3.0 mg.	10 I.M	−(+)
2	−−	Androstendiol	10	9.0 mg.	8 I.M.	−+
2	−−	Androstendione	11	3 0 mg.	10 I.M.	−(+)
2	−−	Androstendione	10	9 0 mg.	8 I.M.	−(+)
2	−−	Pregnenolone	11	3.0 mg	10 I M.	−(−)
2	++†	21-Acetoxypregnenolone	10	9 0 mg.	9 I M.	−−

* 7 days pretreatment with Diethylstilbestrol, 1 mg.
† Untreated.

the zone, and 21-acetoxypregnenolone decreased the stainability from ++ to −−.

It was found that as little as one injection of 3 mgm. testosterone propionate suffices to render a previously negative ICM zone positive, observed eleven days later. It would seem that the phenomenon can be measured quantitatively, as far as the amount and effectiveness of the administered compound is concerned. Three mgm. of methyl testosterone daily for ten days effected almost no change, whereas 9 mgm. daily for nine days achieved the same effect as 3 mgm. testosterone propionate. This 3:1 ratio has also been established in testing the androgenic activity of these compounds by other methods (3). If this same gradient of activity is maintained in other androgenic substances, it is quite possible that the phenomenon of the tissue aldehyde shift might be used for the assay of androgenic activity.

It was thought that stimulation of general metabolic activity might bring about an increase in the tissue aldehyde content of the kidney ICM zone. Table 2 summarizes the results of this second series of experiments. Animals were again pretreated with diethylstilbestrol, then subjected to elevated temperatures of from 32° to 40°C. continuously for five days. No changes in the aldehyde content of the ICM zones were noted. Verne (4) stated that the hyperthermia resulting from injections of dinitrophenol in dogs brought about a greater aldehyde deposition in the kidneys by utilization of fat deposits. Using rats, we could not confirm his results. Injection of foreign protein (staphylococcus toxoid), thyroxine, and adrenaline in oil, 1:500, were likewise without effect in augmenting the aldehyde content of the zone.

The action of some compounds known for their specific action on secretion of urine by the kidney was investigated with a third group of rats. The results are

TABLE 2

The effect of certain metabolically-active drugs and procedure on the intercortico-medullary zone of the rat kidney

NO. OF RATS	ICM ZONE, L. KIDNEY*	SUBSTANCE GIVEN	OBSERVATION PERIOD	DAILY DOSAGE	ADMINISTRATION	ICM ZONE, R. KIDNEY
			days			
2	--		16			--
2	--	Subjected to temp. 32°–40°C.	5		continuous	--
2	--	1% dinitrophenol sodium	11	0.15 cc.	S.C.	--
4	--	1% dinitrophenol sodium	15	0.15 cc.	S.C.	--
2	--	Staphylococcus toxoid	15	0.10 cc.	I.M.	--
2	--	Thyroxine	10	0.33 mg.	I.M.	--
2	--	Adrenalin in oil 1:500	9	0.25 cc.	I.M.	--

* 7 days pretreatment with Diethylstilbestrol, 1 mg.

summarized in table 3. Pitression tannate was selected as representative of an antidiuretic substance. Dosages as high as 0.5 to 1.0 unit per day were without effect on the tissue aldehydes of the ICM zone. It was necessary to administer a dose of 2.5 units per day for nine days, an extremely high dose, to effect a diminution of the stainability of this zone. It was assumed that this effect is due to a suppression of the normally produced testosterone in the animals, which were normal males in this case.

Injections of digitalis and of Mercupurin were ineffective in the dosages given. Theobromine and the various chlorides of sodium, ammonium and calcium, incorporated in the drinking water, also had no effect either in animals with aldehyde depleted or intact ICM zones.

Specificity of the fuchsin sulfurous acid reaction on aldehydes. One may be tempted to question the nature of the substance which is stained with FSA. Perhaps they are not tissue aldehydes, but the products originally injected into the animals. It has been stated that some specific ketones will give a positive reaction with fuchsin sulfurous acid (5). Because of the implications of this

statement in the present work, the specificity of the FSA reagent for aldehydes has been reinvestigated.

Feulgen (6) assumed that the reddish color given by acetone with FSA was not identical with the purple color obtained with aldehydes and therefore named this type of reaction "pseudoreaction." Oster and Mulinos (7) demonstrated that the purple tissue aldehyde-FSA reaction product could be decolorized with alkalis and restored to its original color intensity with acids. In this manner the reaction product behaved as an indicator for strong alkali or acid reaction. This observation has been utilized as a test for true aldehyde-FSA reactions.

TABLE 3

The effect of antidiuretic and diuretic compounds on the intercortico-medullary zone of the rat kidney

NO. OF RATS	SEX	ICM ZONE, L. KIDNEY	SUBSTANCE GIVEN	OBSERVATION PERIOD	DAILY DOSAGE	ADMINISTRATION	ICM ZONE, R. KIDNEY
				days			
1	m	++	Pitressin tannate in oil	15	0.5 un.	12 I.M.	++
2	m	++	Pitressin tannate in oil	15	1.0 un.	12 I.M.	++
2	m	++	Pitressin tannate in oil	11	2.5 un.	9 I.M.	(−)(+)
2	m	++	Digitalis	10	0.5 cat un.	9 I.M.	++
2	m	++	Mercupurin	3†	0.25 cc.	3 I.M.	++
2	m*	++	Theobromine-sodium salicylate	14	2 mg./cc.	In drinking water	++
2	m*	−(+)	Theobromine-sodium salicylate	14	2 mg./cc.	In drinking water	−(+)
3	f*	−− or ++	Sodium chloride	15	1% aq.	In drinking water	No change
3	f*	−− or ++	Ammonium chloride	15	1% aq.	In drinking water	No change
3	f*	−− or ++	Calcium chloride	15	1% aq.	In drinking water	No change

* Castrated.
† Died.

Phenylacetaldehyde, heptylaldehyde anf formaldehyde, as representatives of aromatic and aliphatic aldehydes, gave in vitro the typical purple aldehyde-FSA reaction. Addition of NaOH resulted in declorization and HCl in recolorization of the FSA-aldehyde addition product. Aliphatic ketones such as acetone, methyl ethyl ketone and methyl isobutyl ketone, on the other hand, which gave a reddish coloration with FSA, are not reconstituted to their original color by successive alkalinization and acidification. The final solution remains colorless. If one treats acetone with 1 N HCl, the red colorization with FSA does not appear at all. The same procedure is without any influence, however, on the FSA reaction product obtained with e.g., formaldehyde. Acetophenone, a typical aromatic ketone, effected no reaction whatever with the Schiff's reagent. With-

out going into the theory behind these observations, which may touch on the enol configuration of ketones, the authors regard the alkali-acid test or the acid treatment of ketones as decisive against the argument that ketones give the same reaction with FSA as do aldehydes.

Verne (8) has stated that pure oleic acid does not react with FSA, but that a reaction can be obtained only after shaking the substance in air. Lison (9), on the other hand, obtained a positive reaction with oleic acid. He tested for the absence of aldehydes by using an ammoniacal solution of silver hydroxide (Tollen's Reagent), a test which is not sufficiently fine to detect the minute quantities of aldehydes which would normally give a positive FSA reaction. Oleic acid was tested by us for its reaction with FSA. We could confirm Verne's finding (8) that this compound does not give a positive reaction. Only after simple oxidation with air does the reaction become positive. According to Bloor (10), peroxides are formed in oleic acid which break down to aldehydes, a reaction induced even by exposure to light. Lison (9) further states that cinnamic acid gives a positive FSA reaction. Once more his findings could not be duplicated.

Using phenylhydrazine as reagent, a group of investigators has postulated the presence of ketosteroids in the exact locations where tissue aldehydes have been demonstrated. The validity of this original work by Bennett (11) on the adrenal cortex was challenged by Gomori (12). Verne (8), Motta (13) and Becher (14) demonstrated the presence of aldehydes in Leydig's interstitial cells of the testis and in the ovarian corpus luteum. Pollock (15) and Dempsey and Bassett (16) found phenylhydrazine stainable substances in the identical locations. The latter authors claimed them to be ketosteroids.

Citing unpublished experiments, Dempsey and Wislocki (17) argue that FSA reacts with certain steroids. Therefore, an alcoholic solution of each of the steroids used for injection in the present and previous papers by the authors was subjected to an in vitro test with FSA. The group included both keto- and non ketosteroids. None of them reacted in any way with the reagent. Furthermore, the androgenic steroids devoid of keto groups exerted the same aldehyde depositing influence as those with a keto group. Conversely, progesterone, which is a ketosteroid, consistently effected an aldehyde depletion in the ICM zone.

Dempsey and Wislocki (17) base their conclusions mainly on the work of Lison (9), who claims to have found that positive reactions with FSA could be obtained not only with aldehydes, but also with certain ketones and even with some unsaturated compounds containing no carbonyl group whatsoever. Following the experiments of this author, Dempsey and Wislocki conclude "that these reactions commonly employed to detect aldehydes are not capable of differentiating between aldehydes and ketosteroids." Because of the apparent discrepancies in the interpretation of factual findings, some of the ketones and unsaturated compounds mentioned by Lison (9) were therefore tested by us for their reaction with FSA. His results could not be confirmed.

Without citing additional accumulated evidence, the authors feel secure in concluding that the fuchsin sulfurous acid reaction in tissue sections is given specifically by compounds containing the aldehyde group. When found in tis-

sues, these substances were called tissue aldehydes (2). The reaction is not given by ketosteroids, as claimed by Dempsey and Wislocki (17). By the same argument we believe, in agreement with Gomori (12), that the phenylhydrazones obtained by these authors in tissue sections are formed by the tissue aldehydes and not by ketosteroids and that their findings should be reinterpreted accordingly. Nevertheless, this interpretation should not be so construed as to deny the possible presence of such ketosteroids in the investigated glandular structures. It is merely the histochemical demonstration of these substances which is made doubtful by the methods employed. In quantitative terms also the preponderance of tissue aldehydes in the identical locations of the ketosteroids would make their selective demonstration highly improbable.

CONCLUSIONS

In summarizing these results, it should be emphasized that the disappearance and reappearance of the tissue aldehydes in the intercortico-medullary zone of the rat kidney is governed by the balance of sex hormones. Male sex hormones would seem to be specific for depositing aldehydes and female sex hormones for depleting the aldehydes. Furthermore, we find that the tissue substances stainable with FSA must be aldehydes and not ketosteroids, citing alkaline decolorization and acid recolorization as evidence. One should not overlook the chance that there might be an intermediary product formed in the metabolism of androgenic steroids of aldehydic nature which could give rise to a reaction with FSA. Nevertheless, the possibility of this occurence does seem slim, since excretion of such a product should take place relatively quickly in comparison with the portracted maintenance of the stainable ICM zones after one testosterone propionate injection. Grevenstuk (18) has reported an inability to demonstrate FSA stainable substances in normal urine even after its concentration in vacuo.

It is the belief of the authors, therefore, that the substances giving the observed shift in the ICM zones of the rat kidney are true aldehydes arising in the metabolism of the cell on stimulation by the sex hormones. Only such fatty aldehydes as stearal and palmital have been thus far isolated from tissues. However, other aldehydic compounds of different chemical nature may also be present.

SUMMARY

1. Various influences were tested for their possible effect on the tissue aldehyde content of the rat kidney intercortico-medullary zone, administration of androgenic substances, of antidiuretic and diuretic compounds, of certain electrolytes and stimulation of metabolism. Of these, only androgens effect a deposition of tissue aldehydes in the aldehyde depleted ICM zone.

2. By utilizing the procedure of successive alkalinization and acidification, or acidification alone, proof has been offered that the FSA stainable substances in tissues are true tissue aldehydes and not ketones

3. The work of a group of investigators using phenylhydrazine to demonstrate ketosteroids in tissues may be reinterpreted in the light of the present findings.

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ON THE CHEMOTHERAPEUTIC PROPERTIES OF THE SULFANILANILIDES[1]

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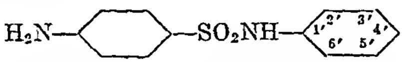
In the course of studies[2] on the antimalarial properties of various sulfanilamide derivatives, a series of sulfanilanilides was synthesized. These compounds with a variety of substituents at the 2′, 3′, 4′, 5′, or 6′ positions had the general structure indicated below.



Marshall and coworkers (1, 2) who studied the activities of these sulfanilanilides against experimental infections with *Plasmodium lophurae*, found that at least two of the more promising derivatives (3′,5′-dibromosulfanilanilide and 3′,5′-dichlorosulfanilanilide) were distinguished from sulfonamides in common use by the fact that their antimalarial activities were not blocked by p-aminobenzoic acid. This finding, coupled with the widespread interest in treatment of infected war wounds and the common belief that necrotic tissue in such wounds abounds in sulfonamide inactivating substances (possibly p-aminobenzoic acid) suggested the desirability of exploring the antibacterial properties of these sulfanilanilides and the influence of p-aminobenzoic acid and tissue breakdown products thereon. Thirty-two of the sulfanilanilide derivatives were made available for this work. All of these compounds were studied in a cursory manner for activity against representative gram-positive and gram-negative bacteria and for their response to p-aminobenzoic acid. A detailed study was made of the properties of one of the most active compounds. The results of these investigations are described here.

I. A SURVEY OF THE CHEMOTHERAPEUTIC PROPERTIES OF A GROUP OF SULFANILANILIDES. METHODS. The following experiments were designed to determine the *in vivo* and *in vitro* activities of the sulfanilanilides against representative species of gram-positive cocci and gram-negative bacilli and to determine the effects of p-aminobenzoic acid on the *in vitro* activities. These studies were carried out in the manner of a screening program and were not in any sense designed to explore the antibacterial activities of all the sulfanilanilides exhaustively.

The test organisms used were pneumococci and Friedlander's bacilli. These organisms

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Institute of Medical Research, The Christ Hospital.

[2] These studies were carried out by various groups of investigators under the direction of the Board for the Coordination of Malarial Studies, Division of Medical Sciences, National Research Council. The results will be summarized in a forthcoming monograph entitled *A Survey of Antimalarial Drugs, 1941–1945*, edited by F. Y. Wiselogle and published by Edwards Brothers, Inc., Ann Arbor, Michigan.

were chosen because they were the only species of gram-positive cocci and gram-negative bacilli available which could be studied satisfactorily in both *in vivo* and *in vitro* tests. The specific organisms employed were the McGovern strain of type I pneumococcus and the GH strain of type A Friedlander's bacillus, strains which have been used in this laboratory for many years.

Determination of in vitro activity. The techniques of carrying out these tests have been described in detail in an earlier publication (3). The following are the essential features. Beef heart infusion broth (4) (enriched with 2 per cent freshly drawn defibrinated rabbit blood on the day prior to inoculation) was used as the basal medium for the tests with pneumococci; a synthetic medium (5), fortified with 0.1 per cent casein hydrolysate (SMACO—vitamin free), was used for the tests with Friedlander's bacilli. Wherever possible, an 89 mgm. per cent solution of the test compound was prepared in these basal media. This solution was diluted serially with equal volumes of the medium to give the desired range of drug concentrations; these were tubed in 9 ml. quantities, autoclaved at 115°C. for 15 minutes and stored in the refrigerator until used. When the inoculum, contained in 1 ml. of basal medium, was added to each tube, the final concentrations of drug in the beef heart medium were 80, 40, 20, 10, 5, 2.5, 1.25, 0.6, and 0.3 mgm. per cent. The final drug concentrations in the semi-synthetic medium were 0.15, 0.08, 0.04, 0.02, and 0.01 mgm. per cent, and those concentrations listed above. When sulfanilanilides were encountered whose solubilities were less than 89 mgm. per cent, which was frequently the case, the highest concentration in the above series at which complete solution was obtained was used for further serial dilution.

In the test procedure pneumococci or Friedlander's bacilli, obtained from mouse passage cultures, were subcultured twice at 12-hour intervals in the appropriate basal medium, the second subculture being used as the source of the inoculum. A 10^{-5} dilution of this subculture was prepared in basal medium, and 1 ml. of this diluted culture was added to each tube of test medium. The number of organisms in this inoculum was determined by plate counts. Visual estimations of growth were made after 12, 24, and 48 hours incubation at 37.5°C. All *in vitro* tests with new compounds were controlled by simultaneous tests with the drugs of reference, sulfanilamide, sulfadiazine and sulfathiazole.

Determination of in vivo activity.. The *in vivo* activities of the sulfanilanilides were evaluated roughly in terms of the activities of sulfanilamide and sulfadiazine. White mice, all of the same stock strain and weighing 14–17 grams, were infected intraperitoneally. In the case of the pneumococci, the infecting dose (approximately 400 organisms) was 1 ml. of a 10^{-6} dilution in broth of a 12–14 hour blood broth culture prepared from the heart blood of a passage mouse. With the Friedlander's bacilli, the infecting dose (approximately 10,000 organisms) was 1 ml. of a 2×10^{-5} dilution in broth of a 12–14 hour blood broth culture, similar to that described above. In the case of either infection, groups of 15 animals received standard doses of the test compound or of the reference drugs, sulfanilamide and sulfadiazine. These doses (2.5 mgm. in pneumococcal infections and 0.5 mgm. in Friedlander's bacillus infections), suspended in 0.2 ml. of a 10 per cent solution of gum acacia, were administered via stomach tube 2, 8, and 14 hours after infection and every 8 hours thereafter for 5 additional days, if the mice survived that long. In each test a group of 10 infected and untreated mice served as controls. Mice surviving the treatment period were observed for 30 days after infection.

Antagonism of in vitro activity by p-aminobenzoic acid. The drug containing media, used in determining the effects of p-aminobenzoic acid on the activity of a compound, were the same as those described above, except for their content of p-aminobenzoic acid. This substance was added in 10 mgm. per cent concentration, an amount twenty to one hundred times larger than that required to completely antagonize the activities of 80 mgm. per cent sulfathiazole against the respective organisms. This concentration of p-aminobenzoic acid was obtained by adding to each tube of medium 0.1 ml. of a one per cent solution neutralized to pH 7.6–7.8. The technique of carrying out the test was identical with that described above for determination of *in vitro* activity.

Evaluation of results. In vitro. Sulfanilamide, the least effective of the reference sulfonamides, was used as the standard of reference and was assigned a value of one. The activities of the sulfanilanilides (or of the other reference drugs, sulfadiazine and sulfathiazole) were determined as the ratio of the minimum concentration of sulfanilamide required to inhibit visible growth for 24 hours to the minimum concentration of the test compound required for the same effect. The inhibitory concentrations of sulfanilamide were 40 mgm. per cent for the pneumococci and 5 mgm. per cent for the Friedlander's bacilli. Thus a test compound which inhibited growth of the pneumococci in a concentration of 1.25 mgm. per cent would be designated as having an activity of 32, while a drug which inhibited growth of Friedlander's bacilli in this same concentration would be designated as having an activity of 4.

In vivo. Both sulfanilamide and sulfadiazine were used as reference drugs in the study of *in vivo* activities. Sulfanilamide, in the dosage employed, prolonged the survival time of infected mice slightly but had essentially no curative action. Sulfadiazine, on the other hand, cured 50 $\pm$ 20 per cent of the infected animals. With these points as reference, the activities of the sulfanilanilides could be expressed as $<$, $=$ or $>$ sulfanilamide or sulfadiazine, depending on the extent to which they prolonged life or possessed curative effects.

Antagonism by p-aminobenzoic acid. In the study of the antagonistic effects of p-aminobenzoic acid, the results were recorded as follows. If p-aminobenzoic acid, in the concentration used, had no effect on activity, results were described as negative (−). If it completely abolished the activity of the test compound throughout the range of available drug concentrations, the results were recorded as positive (+). If the presence of p-aminobenzoic acid decreased the effectiveness of the test drug significantly but did not completely abolish activity, the result was expressed as partial antagonism.

RESULTS. The *in vitro* activities of the sulfanilanilides and the effects of p-aminobenzoic acid on these activities have been recorded in table 1. Examination of this table shows at once that some of the data are incomplete, in that end points were not obtained either in one or both of the activity measurements or in the tests on the effects of p-aminobenzoic acid on activity. Failure to obtain complete data was in every instance due to the low solubilities and activities of the drugs. Irrespective of these limitations, the findings presented in the table are of considerable interest and warrant rather definite conclusions.

In the first place, the data show that many of the sulfanilanilide derivatives possess high *in vitro* activity against the pneumococci. Fifteen of the thirty-two drugs in the series had activities approximating that of sulfathiazole, the most active of the common sulfonamides. Only three of the sulfanilanilide derivatives were definitely less active than sulfanilamide. It is of interest that the unsubstituted sulfanilanilide, the parent compound in the series, had moderate activity, being approximately four times as active as sulfanilamide.

Both the point of substitution and the nature of the substituent appeared to be important factors in determining the *in vitro* activities of the sulfanilanilide derivatives against the pneumococci. Without exception the fifteen most active

TABLE 1

The activities of the sulfanilanilides against pneumococci and Friedlander's bacilli and the effects of p-aminobenzoic acid on these activities

COMPOUND	COMPARATIVE ACTIVITY IN VITRO		ANTAGONISM OF ACTIVITY BY p-AMINOBENZOIC ACID	
	Pneumococcus	Friedlander's bacillus	Pneumococcus	Friedlander's bacillus
Sulfanilamide	1*	1*	+	+
Sulfadiazine	2	32	+	+
Sulfathiazole	16	32	+	+
Sulfanilanilide[a]	4	2	+	+
2′-Chloro-sulfanilanilide[c]	<4	2	n d.†	+
3′-Chloro-sulfanilanilide[c]	8	2	–	+
3′-Bromo-sulfanilanilide[c]	8	1	–	+
3′-Trifluoromethyl-sulfanilanilide[a]	8	$\frac{1}{4}$	–	+
3′-Cyano-sulfanilanilide[b]	8	1	partial	+
3′-Nitro-sulfanilanilide[c]	8	2	partial	+
3′-Methoxy-sulfanilanilide[c]	4	$\frac{1}{2}$	partial	+
3′-Dimethylamino-sulfanilanilide[c]	4	$\frac{1}{4}$	+	+
4′-Chloro-sulfanilanilide[a]	<8	<2	n d.	n.d.†
4′-Trifluoromethyl-sulfanilanilide[a]	<1	$\frac{1}{4}$	n.d.	n.d.
4′-Methoxy-sulfanilanilide[c]	2	$<\frac{1}{4}$	+	n d.
4′-Dimethylamino-sulfanilanilide[c]	<2	$<\frac{1}{4}$	n.d.	n.d.
4′-Tert-butyl-sulfanilanilide[a]	<2	$<\frac{1}{8}$	n.d.	n.d.
4′-Tert-amyl-sulfanilanilide[a]	2	$<\frac{1}{16}$	n.d.	n.d.
2′,5′-Dichloro-sulfanilanilide[c]	<16	<4	n.d.	n.d.
2′-Methoxy,5′-chloro-sulfanilanilide[c]	<8	<1	n.d.	n d.
2′,6′-Dichloro-sulfanilanilide[a]	<1	$<\frac{1}{8}$	n.d.	n.d.
2′,6′-Dibromo-sulfanilanilide[a]	<1	$<\frac{1}{4}$	n.d.	n.d.
3′,4′-Dichloro-sulfanilanilide[c]	<32	$<\frac{1}{4}$	n d.	n.d.
3′-Bromo,4′-tert-butyl-sulfanilanilide[a]	16	$<\frac{1}{2}$	–	n.d.
3′,5′-Dibromo-sulfanilanilide[b]	32	$\frac{1}{2}$	–	+
3′,5′-Trifluoromethyl-sulfanilanilide[a]	32	$<\frac{1}{4}$	–	n.d.
3′,5′-Dicyano-sulfanilanilide[b]	8	$\frac{1}{4}$	partial	+
3′,5′-Dinitro-sulfanilanilide[b]	8	$\frac{1}{2}$	partial	+
4′-Tert-butyl,2′,6′-dibromo-sulfanilanilide[a]	<8	$<\frac{1}{2}$	n.d.	n.d.
4′-Tert-amyl,2′,6′-dibromo-sulfanilanilide[a]	<4	$<\frac{1}{4}$	n d.	n d.

TABLE 1—*Continued*

COMPOUND	COMPARATIVE ACTIVITY IN VITRO		ANTAGONISM OF ACTIVITY BY p-AMINOBENZOIC ACID	
	Pneumococcus	Friedlander's bacillus	Pneumococcus	Friedlander's bacillus
4'-Amino,3',5'-dichloro-sulfanilanilide[a]	8	½	−	+
4'-Dimethylamino,3',5'-dichloro-sulfanilanilide[a]	16	<¼	−	n d.
4'-Amino,3',5'-dibromo-sulfanilanilide[b]	8	¼	−	+
4'-Methylamino,3',5'-dibromo-sulfanilanilide[b]	8	¼	−	+
4'-Dimethylamino,3',5'-dibromo-sulfanilanilide[b]	8	<¼	−	n.d.

* 40 mgm. per cent sulfanilamide was required to inhibit growth of pneumococcus Type I McGovern, 5 mgm. per cent was required to inhibit growth of Friedlander's bacillus Type A. For method of evaluating comparative activities see text.

† n d , non-determinable

[a] These compounds were prepared in the laboratories of Dr. Nathan Drake, Department of Chemistry, University of Maryland, College Park, Maryland.

[b] These compounds were prepared in the laboratories of Dr. Joseph B. Koepfli, Gates and Crellen Laboratories, California Institute of Technology, Pasadena, California.

[c] These compounds were prepared by Dr R G. Shepherd of the Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, Maryland.

drugs were substituted at the 3' or the 3' and 5' positions. Chloro-, bromo- or trifluoromethyl-substituents were the most effective. Also without exception, the eleven least active sulfanilanilides were substituted at the 2', the 4', the 2',6' or the 2',4',6' positions. Interestingly enough, chloro-, bromo- or trifluoromethyl-substituents at these latter positions did not enhance activity.

In contrast to their high activity against the pneumococci, all of the sulfanilanilides possessed comparatively low activity against the Friedlander's bacilli. Only six of the derivatives were as active as sulfanilamide and the best of these was not more than twice as active as this sulfonamide and no more than one-sixteenth as active as sulfathiazole or sulfadiazine. Twenty-three of the derivatives were definitely less active than sulfanilamide. Interestingly, the parent unsubstituted sulfanilanilide was one of the most active compounds, and in contrast to the findings with the pneumococci, substitution at almost any position reduced the activity of the parent drug.

Especially noteworthy were the effects of p-aminobenzoic acid on the activities of the sulfanilanilides. In every instance where determinations could be made, p-aminobenzoic acid blocked the activities of these compounds against Friedlander's bacilli. Thus, with this organism, the sulfanilanilide-p-aminobenzoic acid relationship was the same as that which has been observed with the common

sulfonamides. This relationship did not hold with the pneumococci, however. The antipneumococcal activities of eleven derivatives were not in the least affected by 10 mgm. per cent p-aminobenzoic acid, and the activities of five other compounds were only partially antagonized. With only three compounds were antipneumococcal properties completely blocked. One of these substances was the unsubstituted sulfanilanilide, the parent compound of the series; a second was the 3′-dimethylamino-derivative; the third was the 4′-methoxy-derivative.

It is clear from the data in the table that the nature of the substituent was a primary factor in determining the effects of p-aminobenzoic acid on the antipneumococcal activities of sulfanilanilides. Thus, the activities of compounds with 3′, or 3′,5′-chloro-, bromo- or trifluoromethyl-substituents were not at all affected. Activities of compounds with 3′ or 3′,5′-nitro- or cyano- or 3′-methoxy-substituents were partially antagonized by p-aminobenzoic acid, whereas the activity of the 3′-dimethylamino-derivative was completely blocked.

Whether the point of substitution was also a factor in the sulfanilanilide-p-aminobenzoic acid relationship could not be determined from the data at hand. The fact that p-aminobenzoic acid only partially blocked the activity of the 3′-methoxy-derivative but completely blocked the activity of the 4′-methoxysulfanilanilide suggests that the point of substitution may be a factor, but these data are too few to support such a generalization.

The *in vivo* activities of the sulfanilanilides need not be described or discussed in detail inasmuch as, at the doses employed, none of the drugs displayed a significant degree of activity against the test organisms. None of the compounds was more active than sulfanilamide against infections with either pneumococci or Friedlander's bacilli, and only the 3′,5′-trifluoromethyl-derivative had as great activity as this sulfonamide.

II. Detailed study of the chemotherapeutic properties of 3′,5′-dibromosulfanilanilide. A detailed investigation was made of the antibacterial properties of 3′,5′-dibromosulfanilanilide, one of the most active of the compounds discussed in the earlier part of this report. In this study, attention was focussed on: (1) the activity of the drug *in vitro* against various species of gram-positive cocci and gram-negative bacilli; (2) its activity against strains of pneumococci and staphylococci which had acquired resistance to the common sulfonamides; (3) the effects of p-aminobenzoic acid and pus on the activity of the drug against various bacterial species; and (4) a wider exploration of its activity against experimental infections than was carried out in the preliminary study described above.

Methods. *In vitro studies.* The organisms used in this study included five strains of sulfonamide-sensitive and four strains of sulfonamide-resistant pneumococci, two strains of β-hemolytic streptococci, five strains of sulfonamide-sensitive and nine strains of sulfonamide-resistant staphylococci, one strain each of Friedlander's bacilli, *Escherichia coli* and *Shigella ambigua* and two strains each of *Pseudomonas aeruginosa*, *Shigella paradysenteriae* and *Shigella sonnei*[3]. The stock cultures of pneumococci, β-hemolytic streptococci, Fried-

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[3] We are indebted to Dr. Merlin Cooper, Children's Hospital Research Foundation, Cincinnati, Ohio, for the cultures of dysentery bacilli; to Mrs. Rosemary Bole, Department of

lander's bacilli and certain of the staphylococci were maintained by daily mouse passage. The stock cultures of the remaining staphylococci were maintained by transfer every other day in meat infusion broth, the strains of *Pseudomonas aeruginosa* and *Escherichia coli*, by weekly passage in meat infusion broth, and the strains of dysentery bacilli, by weekly transfer in semi-solid medium (meat infusion broth containing 0.2 per cent agar).

The basal media employed in this study were the same as those described in part I with the addition of Knight's medium (6), essentially as modified by Strauss et al. (7), for some of the tests with staphylococci. The techniques of preparing the drug containing media, the preparation of the cultures to be used in the activity measurements and the actual set-ups of the experiments were essentially the same as those described in the preceding section, with the exception that the drug was incorporated in 8 ml. quantities of medium for the tests with p-aminobenzoic acid and pus, in order that these substances could be added in 1 ml. volumes to each tube, making the final volume 10 ml. after addition of the inoculum

The pus used in this study was obtained from eight different patients representing a variety of lesions and infecting organisms. Various methods of preparation of the pus were used including filtration through muslin, autolysis, and extraction with heat, acids, alkalis or ether. The preparations were divided and portions sterilized by boiling, autoclaving, or Seitz filtration Varying amounts of these sterilized materials, contained in 1 ml of solution, were added to the tubes of test media.

In all of the *in vitro* studies parallel experiments were carried out with the 3′,5′-dibromosulfanilanilide and sulfathiazole.

In vivo studies *In vivo* experiments were carried out with the McGovern strain of type I pneumococcus and the C203 strain of β-hemolytic streptococcus. The techniques of infection and treatment were the same as those described in part I except that varying doses of the 3′,5′-dibromosulfanilanilide were administered and the duration of treatment in the experiments with β-hemolytic streptococci was three days In all experiments the effectiveness of the 3′,5′-dibromosulfanilanilide was compared with that of sulfadiazine

Results. *Activities against various gram-positive cocci.* The *in vitro* activities of the 3′,5′-dibromosulfanilanilide against various strains of pneumococci, β-hemolytic streptococci and staphylococci have been summarized in table 2. It is readily apparent from the data presented that in essentially all instances the activities of 3′,5′-dibromosulfanilanilide against these organisms were as great as those of sulfathiazole and under some conditions were strikingly greater. The two drugs had essentially identical activities against the five strains of pneumococci, but the sulfanilanilide was at least sixty-four times as active as sulfathiazole against the β-hemolytic streptococci. The test conditions determined the relative effectiveness of the drugs against the staphylococci. When the tests were carried out in Knight's semi-synthetic medium, activities were essentially the same. However, when the tests were performed in complex medium, the sulfanilanilide was from sixteen to one hundred twenty-eight times as active as sulfathiazole. This difference in relative activities was due to the fact that substantially more sulfathiazole was required to inhibit growth in the complex medium than in the synthetic medium, whereas the effective concentrations of the sulfanilanilide were the same in both media. It should be remarked here that in contrast to the results with sulfathiazole there was very little variation in the amounts of the sulfanilanilide required to inhibit growth of any of the

Bacteriology, Ohio State University, Columbus, Ohio, for the *Pseudomonas aeruginosa* strains, and to Dr Wesley Spink, Division of Internal Medicine, University of Minnesota Hospitals and Medical School, for the majority of the strains of *Staphylococcus aureus*.

seventeen different organisms. Thus, the effective concentrations of the sulfanilanilide for these organisms varied only fourfold, whereas those of sulfathiazole varied more than five hundredfold.

Activities against various gram-negative bacilli. As contrasted with its activity against the gram-positive cocci, 3′,5′-dibromosulfanilanilide possessed relatively low activity against such gram-negative bacilli as Friedlander's bacilli,

TABLE 2

Comparative activities of 3′,5′-dibromosulfanilanilide and sulfathiazole against various strains of pneumococci, β-hemolytic streptococci and staphylococci

ORGANISM	TEST MEDIUM	DRUG REQUIRED TO INHIBIT GROWTH FOR 24 HOURS	
		3′,5′-Dibromosulfanilanilide	Sulfathiazole
		mgm. per cent	*mgm. per cent*
Pneumococcus			
McGovern strain (Type I)	Beef heart broth + 2% blood	1.25	2.5
SV-1 strain (Type I)		2.5	1.25
CH strain (Type II)		1.25	1.25
CHA strain (Type III)		1.25	1.25
Wistuba strain (Type III)		1.25	2.5
β-Hemolytic streptococcus			
CF-1 strain	Beef heart broth + 2% blood	1.25	80
C203 strain		2.5	>160
Staphylococcus aureus			
679 strain	Beef heart broth	2.5	40
Smith strain		2.5	40
7 strain		2.5	160
14 strain		2.5	160
Eden strain		0.6	80
679 strain	Knight's semi-synthetic medium	0.6	0.3
Smith strain		0.6	1.25
7 strain		1.25	2.5
14 strain		0.6	0.6
Eden strain		1.25	1.25

Escherichia coli, *Pseudomonas aeruginosa* and dysentery bacilli (cf. table 3). In every instance where a complete assessment of activity could be made, the sulfanilanilide was less active than sulfathiazole. Thus, in the semi-synthetic medium the sulfanilanilide possessed only one sixty-fourth to one sixteenth the effectiveness of sulfathiazole. There was some variation in the activity of the sulfanilanilide against the different organisms, the drug being distinctly less effective against *Pseudomonas aeruginosa* than against the other organisms. It is especially noteworthy that the concentrations of the sulfanilanilide required for growth inhibition in the complex medium were substantially greater than those in the simple medium, this finding being in marked contrast to the results obtained with the staphylococci (cf. table 2).

Effects of p-aminobenzoic acid on activity. The effects of p-aminobenzoic acid on the activities of 3′,5′-dibromosulfanilanilide and sulfathiazole against the various bacterial species have been summarized in table 4. The data presented there show that the activities of the sulfanilanilide against pneumococci, β-hemolytic streptococci and staphylococci were affected little, if at all, by p-

TABLE 3

Comparative activities of 3′,5′-dibromosulfanilanilide and sulfathiazole against various gram-negative bacilli

ORGANISM	TEST MEDIUM	DRUG REQUIRED TO INHIBIT GROWTH FOR 24 HOURS	
		3′,5′ Dibromosulfanilanilide	Sulfathiazole
		mgm per cent	*mgm per cent*
Friedlander's bacillus			
GH strain	Sahyun	10	0 16
Escherichia coli			
CH strain	Sahyun	2 5	0 16
	Beef heart	>20	80
Pseudomonas aeruginosa			
PA strain	Sahyun	>20	1 25
Chicago strain	Sahyun	>20	1 25
Shigella ambigua			
S200 strain	Sahyun	5	0 16
	Beef heart	>20	20
Shigella paradysenteriae			
Bennett strain	Sahyun	1 25	0 01
Bennett strain	Beef heart	20	5
Mudd strain	Sahyun	1 25	0 02
Mudd strain	Beef heart	10	0 6
Shigella sonnei			
Cheatam strain	Sahyun	2 5	0 16
Cheatam strain	Beef heart	>20	80
Martin strain	Sahyun	10	0 16
Martin strain	Beef heart	>20	80

aminobenzoic acid, whereas this substance blocked the activity of the drug against Friedlander's bacilli, *Escherichia coli* and dysentery bacilli. It should be noted that these differences in reactivity to p-aminobenzoic acid were not merely a matter of quantitative susceptibility for even 100 mgm. per cent of this substance did not block the activity of the 3′,5′-dibromosulfanilanilide against the gram-positive cocci, whereas 10 mgm. per cent or less sufficed to block activity against the gram-negative bacilli. It should also be pointed out that in keeping with the findings of many investigators, p-aminobenzoic acid blocked the activities of sulfathiazole against all the organisms employed in this study.

Activity against sulfonamide-resistant pneumococci and staphylococci. The failure of p-aminobenzoic acid to block the activities of 3′,5′-dibromosulfanilanilide against the pneumococci and staphylococci, together with the alleged relationship between sulfonamide resistance and excessive production of p-amino-

TABLE 4

The effects of p-aminobenzoic acid on the activities of 3′,5′-dibromosulfanilanilide and sulfathiazole

ORGANISM	TEST MEDIUM	p-AMINOBENZOIC ACID	DRUG REQUIRED TO INHIBIT GROWTH FOR 24 HOURS	
			3′,5′-Dibromosulfanilanilide	Sulfathiazole
		mgm. per cent	*mgm. per cent*	*mgm. per cent*
Pneumococcus Type I McGovern strain	Beef heart broth + 2% blood	0	2.5	5
		0.1	2.5	80
		1	2.5	>160
		10	2.5	>160
		100	1.25	>160
β-Hemolytic streptococcus CF-1 strain	Beef heart broth + 2% blood	0	2.5	40
		1	5	80
		10	5	>160
		100	2.5	>160
Staphylococcus aureus No. 7 strain	Knight's semi-synthetic medium	0	1.25	2.5
		1	5	160
		10	5	>160
Friedlander's bacillus GH strain	Sahyun's semi-synthetic medium	0	2.5	0.16
		10	20	160
Escherichia coli CH strain	Sahyun's semi-synthetic medium	0	2.5	0.16
		10	>20	>160
Shigella ambigua S209 strain	Sahyun's semi-synthetic medium	0	2.5	0.08
		10	>20	160
Shigella paradysenteriae Bennett strain	Sahyun's semi-synthetic medium	0	2.5	0.08
		10	>20	160
Shigella sonnei Martin strain	Sahyun's semi-synthetic medium	0	2.5	0.16
		10	>20	160

benzoic acid (8–12), made it of special interest to determine the effectiveness of the sulfanilanilide against sulfonamide-resistant organisms. Such a study was carried out with the results shown in table 5. The data presented there show that four sulfonamide-resistant strains of pneumococci and three resistant strains of staphylococci were as susceptible to the sulfanilanilide as the sulfonamide-sensitive strains from which they were derived. Furthermore, the dibromo-

sulfanilanilide was as active against six other sulfathiazole-resistant strains of staphylococci as against any of the sulfonamide-sensitive strains.

The effects of pus on activity. As stated previously, pus from eight different patients was used in this study. In all, twenty-one different preparations of

TABLE 5

The activities of 3′,5′-dibromosulfanilanilide and sulfathiazole against sulfonamide-resistant strains of pneumococci and staphylococci

ORGANISM	DRUG REQUIRED TO INHIBIT GROWTH FOR 24 HOURS	
	3′,5′-Dibromosulfanilanilide	Sulfathiazole
	mgm per cent	*mgm per cent*
*Pneumococcus**		
Type I McGovern parent strain	1.25	2 5
Type I McGovern resistant strain	2.5	80
Type II CH parent strain	1 25	2 5
Type II CH resistant strain	2 5	80
Type III Wistuba parent strain	1.25	5
Type III Wistuba resistant strain	2 5	160
Type III CHA parent strain	2 5	5
Type III CHA resistant strain	2 5	160
Staphylococcus aureus†		
No 7 parent strain	2 5	2 5
No. 7 resistant strain	5	80
No 14 parent strain	2 5	<0.6
No. 14 resistant strain	5	40
Eden parent strain	1.25	2 5
Eden resistant strain	2 5	160
No 604 resistant strain	5	80
No. 605 resistant strain	2 5	>160
No. 606 resistant strain	5	160
No. 614 resistant strain	2 5	>160
No 616 resistant strain	2 5	>160
No 628 resistant strain	2 5	>160

* Tests with pneumococci were carried out in beef heart medium enriched with 2% blood.
† Tests with staphylococci were carried out in Knight's medium.

these materials were examined for their effects on the activities of 3′,5′-dibromosulfanilanilide against the pneumococci, staphylococci, β-hemolytic streptococci and *Escherichia coli*—parallel experiments being carried out with sulfathiazole. The results of this study will only be mentioned here since they will be described in detail in a separate communication. Summarized briefly, the results showed

that none of the twenty-one preparations was able to block the activities of the sulfanilanilide against the pneumococci, staphylococci or β-hemolytic streptococci, whereas eighteen of these preparations effected a significant reduction in the activity of the drug against *Escherichia coli.*

It should be pointed out that essentially the same pattern of result was obtained in control experiments with sulfathiazole. This finding, which is completely at odds with the widely accepted conception that pus and tissue breakdown products inhibit the activities of the common sulfonamides, will be discussed in the more complete report of this work.

Activity against experimental infections with β-hemolytic streptococci and pneumococci. Experiments carried out earlier in this study (cf. Part I) showed that 3′,5′-dibromosulfanilanilide at 2.5 mgm. dosage had little effect on the course of experimental pneumococcal infections, having no more effect than sulfanilamide and distinctly less than sulfadiazine. Whereas this finding suggested rather plainly that the sulfanilanilide would have little place in the treatment of systemic infections, it seemed well not to dismiss this possibility entirely, merely on the basis of the screening study. Accordingly, the *in vivo* activities of the drug against pneumococcal and streptococcal infections were explored more extensively, using a wider range of doses.

The results of the experiments with pneumococci will not be detailed here, for within a dosage range of 2 to 64 mgm., 3′,5′-dibromosulfanilanilide failed to show any beneficial action on the course of the experimental infection. Actually, the higher doses of the drug had the opposite effect and shortened the survival times of the treated mice significantly over those of the untreated controls.

The results of the experiments with the streptococci have been summarized in table 6. The data there show that doses of 2, 4 and 8 mgm. had a slightly beneficial effect on the course of the infection, but this effect was considerably less than that obtained with 0.5 mgm. doses of sulfadiazine. Doses larger than 8 mgm. were in themselves toxic. At 64 mgm. the beneficial effects of the drug were lost, the treated mice dying more rapidly than the untreated controls.

It is worth pointing out here that even doses of the dibromosulfanilanilide which were toxic produced comparatively low blood levels. Thus, at doses of 10 mgm. the peak levels of the free drug averaged only 4.5 mgm. per cent. This figure is approximately that obtained under the same conditions with one twentieth the dosage of sulfadiazine.

Discussion. The results of the *in vitro* studies presented above have shown that there are many features of theoretical interest in the antibacterial activities of the sulfanilanilides. These features involve the differential effectiveness of these drugs against various bacterial species, the relationship of chemical structure to activity against a given organism, the unique response of at least some of the drugs to antagonism by p-aminobenzoic acid and other substances which are commonly regarded as sulfonamide inhibitors, and finally, the effectiveness of at least one of the compounds against sulfonamide-resistant organisms.

In connection with the first of these points, it was found that as compared with such sulfonamides as sulfanilamide, sulfadiazine, and sulfathiazole, the sulfa-

nilanilides as a group possess relatively much greater activities against the pneumococci than against Friedlander's bacilli. Further experiments with a representative sulfanilanilide (3′,5′-dibromosulfanilanilide) showed that this compound also possesses high activity against other gram-positive cocci such as the staphylococci and β-hemolytic streptococci but has little activity against such other gram-negative bacilli as *Escherichia coli, Pseudomonas aeruginosa* and dysentery bacilli. This finding suggests that relatively high activities against gram-positive cocci and low activities against gram-negative bacilli are general characteristics of the sulfanilanilides.

TABLE 6

Activity of 3′,5′-dibromosulfanilanilide against experimental infections with β-hemolytic streptococci, strain C203

(Infecting dose 490 organisms—15 mice in each group)

DRUG	DOSE	NUMBER OF DEATHS DAYS AFTER INFECTION								SURVIVAL OF MICE THAT DIED	SURVIVORS	
		1	2	3	4	5	6	7–10	11–30		No	Per cent
	mgm *									*hours*		
0		15	0	0	0	0	0	0	0	17	0	0
Sulfadiazine	0.5	0	0	0	0	0	4	5	0	157	6	40
	1.0	0	0	0	0	0	1	5	2	210	7	47
	2.0	0	0	0	0	0	0	2	3	273	10	67
3′,5′-Dibromo-sulfanilanilide	2	0	12	1	2	0	0	0	0	39	0	0
	4	1	5	0	5	4	0	0	0	71	0	0
	8	1	1	2	9	2	0	0	0	82	0	0
	16	4	8	0	2	1	0	0	0	43	0	0
	32	9	6	0	0	0	0	0	0	25	0	0
	64	15	0	0	0	0	0	0	0	14	0	0

* Indicated dose administered at 2, 8, and 14 hours after infection and every 8 hours thereafter for 5 additional doses, or as long up to that time as the mice survived

The above finding is opposed to the common impression that sulfonamides lack specificity of action and possess the same relative orders of activity against different bacterial species (13-19). It is noteworthy that the evidence in support of this impression is not too conclusive and there was some prior evidence (3, 20) in opposition. It might also be pointed out that the sulfanilanilides resemble the sulfones in possessing relatively high activities against gram-positive cocci and low activities against gram-negative bacilli (21).

The correlation between the antipneumococcal activities and chemical structures of the sulfanilanilides is striking. Thus, derivatives with substituents at the 3′ or 3′,5′ positions (and particularly halogen substituents) possess considerably higher antipneumococcal activities than derivatives with substituents at other positions. The precise explanation of this finding is not apparent, but its implications may be highly important for the development of new and more active drugs in the sulfanilanilide series.

One of the most remarkable findings in the present study was the response of the sulfanilanilides to p-aminobenzoic acid. The activities of these compounds against Friedlander's bacilli are blocked by p-aminobenzoic acid, just as are the activities of such conventional sulfonamides as sulfanilamide, sulfathiazole, and sulfadiazine. However, unlike this classical relationship, the activities of many of the sulfanilanilides against the pneumococci are either completely unaffected by p-aminobenzoic acid or are blocked only to a slight degree. Experiments with 3′,5′-dibromosulfanilanilide showed that this peculiar behavior was not limited to the two organisms mentioned above but applied in the same pattern to other gram-positive cocci and gram-negative bacilli.[4] These reactions of the sulfanilanilides are thus distinctly different from either those of the common sulfonamides or of the homosulfonamides (whose activities against both gram-positive cocci and gram-negative bacilli are unaffected by p-aminobenzoic acid (18, 24).

In connection with the antagonism of sulfanilanilide activity it should be noted that the reactions of the 3′,5′-dibromosulfanilanilide to the constituents of complex media parallel the reactions of this drug to p-aminobenzoic acid. Activities of this sulfanilanilide against gram-negative bacilli were blocked by constituents of complex media; activities against gram-positive cocci were not affected.

The response of sulfonamide-resistant staphylococci and pneumococci to 3′,5′-dibromosulfanilanilide is of special interest, in view of the indifference of this drug to p-aminobenzoic acid and the implication that excessive production of this substance is responsible for sulfonamide resistance (8–12). The finding that the activities of the above sulfanilanilides are as great against sulfonamide-resistant organisms as against the parent sensitive organisms would appear to support the above implication. The explanation may not be as simple as this, however. Excessive production of p-aminobenzoic acid or arylamine (10, 11) characterizes some, if not all, of the strains of resistant staphylococci used in the present study but on the other hand the resistant pneumococci produce no more p-aminobenzoic acid or other sulfonamide inhibitors than do the parent strains (10). It may well be that the effectiveness of 3′,5′-dibromosulfanilanilide against these resistant organisms is entirely independent of its indifference to p-aminobenzoic acid and the drug affects these organisms via different mechanisms than do the common sulfonamides. Also, the possibility exists that the sulfanilanilides attack the staphylococci via one mechanism and the pneumococci via another.

[4] Goetchius and Lawrence (22), in a recent publication appearing after the present experiments had been completed, drew the broad generalization that the antibacterial activities of the sulfanilanilides are unaffected by p-aminobenzoic acid. This conclusion was based on experiments with only one sulfanilanilide, the 3′,5′-dibromo-derivative, and only one organism, the β-hemolytic streptococcus. In view of the observations reported in the present paper, this generalization of Goetchius and Lawrence seems unwarranted and requires considerable qualification both with respect to the character of the sulfanilanilide and the species of microörganism. This conclusion is supported not only by our own work but by the completely independent observations of White (23).

The features mentioned above appear difficult to reconcile with the current conception that the sulfonamides owe their activities to their ability to compete with p-aminobenzoic acid. Where the sulfanilanilides possessed their highest activity, as against the gram-positive cocci, there was no antagonism by p-aminobenzoic acid. On the other hand, where activities were low, as against the gram-negative bacilli, there was blocking of activity by p-aminobenzoic acid. These findings suggest that the activities of these drugs are not solely dependent upon their competition with p-aminobenzoic acid and, further, that their activities against gram-positive cocci may depend upon entirely different mechanisms from those which affect the gram-negative bacilli. Further exploration of this apparent dualistic action of the sulfanilanilides would seem to be indicated since it might not only be of use in explaining the modes of action of the sulfanilanilides but also assist in explaining the activities of the common sulfonamides.

Finally it should be pointed out that on the basis of the *in vivo* studies reported here there would appear to be little use for any of the present group of sulfanilanilides in the treatment of systemic bacterial infections. The poor absorption of the best of these drugs coupled with comparatively high toxicity makes them inferior to such a drug as sulfadiazine. Theoretically, at least, the indifference of some of the sulfanilanilides to antagonism by tissue breakdown products and p-aminobenzoic acid might make these drugs of some use in certain local infections with gram-positive organisms. Even such usage does not appear promising, in view of the low activities of the sulfanilanilides against the gram-negative bacilli which so often are a part of the flora in such lesions. Certainly local use should not be attempted until a careful investigation has been made of tissue reactions to these drugs.

SUMMARY

A series of thirty-two sulfanilanilide derivatives have been examined for *in vitro* activities against pneumococci and Friedlander's bacilli and for their effectiveness against experimental infections with these organisms, with the following results *In vitro*, nearly all of these sulfanilanilides possessed considerable activity against the pneumococci. Compounds with halogen, cyano- or nitro-groupings in the 3′ or 3′,5′ positions were most active. None of the sulfanilanilides possessed significant *in vitro* activities against the Friedlander's bacilli. None of the compounds demonstrated significant activity against experimental infections with either of the test organisms

Following a lead obtained in antimalarial investigations, a considerable number of the sulfanilanilides were studied for response to p-aminobenzoic acid. It was found that the antipneumococcal activities of numerous compounds were either not affected at all or were only partially blocked by p-aminobenzoic acid. Compounds reacting in this manner included those with 3′ or 3′,5′-chloro-, bromo-, trifluoromethyl-, nitro-, cyano-, or methoxy-substituents. In contrast to this, p-aminobenzoic acid uniformly blocked the activities of the sulfanilanilides against the Friedlander's bacilli.

A more extensive study of *in vitro* and *in vivo* characteristics of the sulfanilani-

lides was carried out using, as the test drug, 3′,5′-dibromosulfanilanilide, one of the most active of these compounds. The results of the *in vitro* studies showed that this substance possessed considerable activity against numerous strains of pneumococci, β-hemolytic streptococci and staphylococci, but had little activity against Friedlander's bacilli, *Escherichia coli*, *Pseudomonas aeruginosa* and various types of dysentery bacilli. Interestingly, the drug was equally active against sulfonamide-sensitive and resistant pneumococci and staphylococci Furthermore, its activities against the various gram-positive cocci were not affected by p-aminobenzoic acid whereas activities against the gram-negative bacilli were uniformly blocked. The same general relationship held for antagonism of activity by the constituents of complex media or pus

The *in vivo* studies showed that the 3′,5′-dibromosulfanilanilide exerted little favorable effect on the course of experimental infections with β-hemolytic streptococci and none whatsoever on infections with pneumococci. The compound displayed considerable toxicity.

The practical and theoretical implications of these findings have been discussed.

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THE FATE OF ACETYLSALICYLIC ACID

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Salicylates, particularly as acetylsalicylic acid, are among the most widely used therapeutic agents. In the last decade the concentration of salicylates appearing in the blood and the rate of excretion after massive doses, such as used in the treatment of rheumatic fever, have been studied. Little information, however, has been obtained as to these features for the doses taken in the much wider use of salicylates as analgesics and antipyretics by nonrheumatic individuals.

The investigation reported here deals with these smaller doses and includes a study of 1) the excretion; 2) the forms and concentrations in which salicylates appear in the plasma; the influences of bicarbonate upon absorption, plasma concentration and elimination; and accumulation of salicylate; 3) the binding of salicylates by plasma; 4) the distribution of salicylates in body fluids; 5) the nature of renal excretion of salicylates; and 6) a discussion of the analgesia.

Analytical methods. The method of Brodie, Udenfriend and Coburn (1), modified as described here, was used to determine salicylates in plasma and whole blood. Larger volumes of plasma, blood and extractants were used than called for in the original description; the sensitivity of the method was thus increased so that it was possible to detect as little as 0.04 mg. per cent of salicylic acid. To obtain this sensitivity, 8 cc. of the fluid under analysis were made acid with 2 cc. 6N HCl and extracted with 24.5 cc. of ethylene dichloride; 20 cc. of the ethylene dichloride were then re-extracted with 2 cc. of water and 0.10 cc. of 0.5 per cent ferric chloride. The aqueous extract was transferred to a flat-bottomed microcolorimeter tube and its optical density determined with a Klett colorimeter using a green filter transmitting light of 540 mμ. With these relative volumes a concentration of 1 mg. per cent salicylic acid in the original 8 cc. sample gives a reading of 109 divisions on the colorimeter scale. A calibration curve was obtained for volumes less than 8 cc. using 0.5 cc. 6N HCl for each 2 cc. portion of fluid to be analyzed and keeping the other volumes constant; the resulting relationship between divisions per milligram per cent and volume of fluid analyzed is not linear since ethylene dichloride does not extract the salicylic acid completely from the aqueous solution and changes in relative volumes therefore exert effects disproportionate to the volume of fluid extracted.

It was found that whole blood could be analyzed as easily as plasma by this technique. The method was also used satisfactorily for the determination of salicylate in cerebrospinal fluid and ultrafiltrate of plasma.

Conjugated salicylate in both whole blood and plasma can be determined by the hydrolysis of the material in a solution 2N in HCl using the reflux apparatus[1] described by Greenberg and Lester (2). It is thus possible, as discussed later, to determine acetylsalicylate by difference.

As indicated by Brodie, Udenfriend and Coburn, free salicylate in nonhydrolyzed urine cannot be determined by their method, but it has been found here that it may be used for total salicylate in hydrolyzed urine. Prior to the publication of this method total urinary

[1] Made by the Macalaster Bicknell Co., New Haven, Conn.

salicylate determinations were made by us using the following method, which is highly satisfactory for this purpose.

A volume of urine, acidified to be 2.6N in sulfuric acid, is hydrolyzed by refluxing for 60 minutes; this strength of acid and length of time suffice to hydrolyze all conjugates of salicylate including salicylurate; determinations made at longer times give no increase of salicylate. The hydrolysate is extracted with an equal volume of diethyl ether; a suitable aliquot of the ether is evaporated slowly to dryness to minimize mechanical carry-over of salicylic acid; the residue is dissolved in water, ferric chloride is added and the optical density of the solution is then determined. Calibration, recovery of known amounts from urine, and blanks were determined in the usual manner. This method is both accurate and sensitive and was used for most of the analyses of total urinary salicylate reported here.

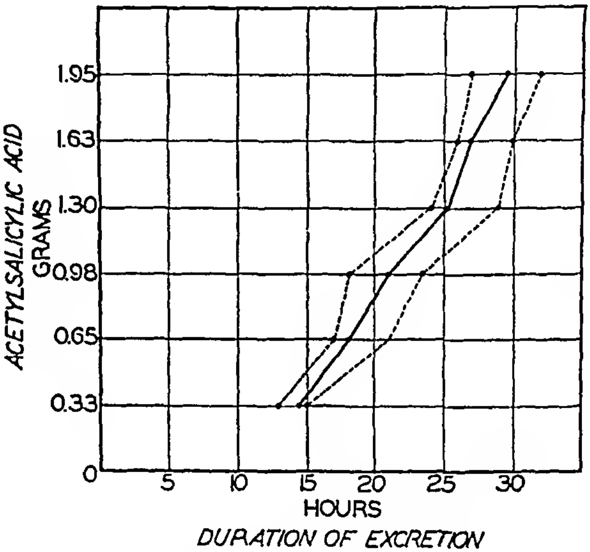

FIG. 1. RELATION BETWEEN VARIOUS DOSES OF ACETYLSALICYLIC ACID AND TIME OF EXCRETION
●—●—●: Extremes of duration of excretion. O—O—O: Average duration of excretion.

EXCRETION. Human male subjects were employed; they were mainly laboratory personnel in good health. Blood was obtained from the antecubital vein with heparin[2] as the anticoagulant.

The duration and amount of excretion of salicylate in the urine were determined after oral administration of 0.33 to 1.95 g. of acetylsalicylic acid. Seven subjects took a dose of 0.33 g.; 6 took 0.65, 0.98 and 1.30 g.; and 5 took 1.63 and 1.95 g. The end-point of excretion was taken arbitrarily as the time when less than 5 mg. of salicylate were excreted per hour; at this time about 95 per cent of the total excretion had occurred. The acetylsalicylic acid was administered at

[2] Supplied by the Roche-Organon Co.

such times that the end-points would fall within the working day so that hourly samples might be more easily procured. There was thus no fixed relation in time between food intake and ingestion of the acetylsalicylic acid. Figure 1 shows the results obtained.

As will be shown later, acetylsalicylate appears in the plasma in very small amounts and for a very short time immediately after ingestion of the drug, while free salicylate after this first period, constitutes all the salicylate in the plasma. This suggested, therefore, that the relative amounts of salicylate compounds excreted after acetylsalicylic acid would be the same as those excreted after sodium salicylate as found by Kapp and Coburn (3). To test this, several urine specimens were analyzed for the relative amounts of salicylurate and free salicylate. The nonhydrolyzed acidified urine was extracted with two equal volumes of ether, which extracts all the salicylic acid and about 96 per cent of the salicyluric acid. Hydrolysis of an aliquot of this extract and determination of glycine (4, 5) in the hydrolysate gives the amount of salicyluric acid. By determination of the total color developed from the ether extract with ferric chloride, and knowing the contributions to the color of salicylic and salicyluric acids and the amount of salicyluric acid from glycine analysis, free salicylic acid is given by difference. In this manner it was found that free salicylate constituted some 20 to 25 per cent and salicylurate some 50 to 55 per cent of the total salicylate excreted in the samples investigated, which corresponds to the values found by Kapp and Coburn (3).

In the series of 35 determinations reported here from 52 to 75 per cent of the amount of salicylate ingested was excreted in the urine; the average was 68 per cent. Variations in per cent excreted showed no correlation to dosage or urinary volume. Within the range of extremes the same individual varied in different experiments and these variations likewise showed no relation to variations in dosage and urinary volume.

Figure 2 shows representative curves of the hourly excretion of salicylates after administration of various amounts of acetylsalicylic acid.

The literature affords little data on the duration of excretion after small doses. Blanchier (6) found excretion to be practically complete in 22 hours after 1 to 2 g. of sodium salicylate, while Hanzlik, DeEds and Presho (7) reported that after 0.5 to 1.0 g. of various salicylates, excretion of detectable amounts continued for 48 hours. The present data are in accord with Blanchier's findings.

Salicylates in the plasma. From the duration of excretion it is evident that even after the ingestion of small amounts of acetylsalicylic acid, salicylate persists in the plasma for many hours. Typical curves of the concentration of salicylate in the plasma for doses of 0.65 and 1.30 g. to an individual weighing 75 kg. are shown in figure 3 as A and A′. The maximum concentration reached after ingestion of 0.65 g. was of the order of 4 mg. per cent and after 1.30 g., 8 mg. per cent. These concentrations are for free salicylate only and do not include acetylsalicylate which may be present.

If some acetylsalicylate is absorbed without alteration the probability that it will disappear from the plasma in a short time after absorption is indicated by

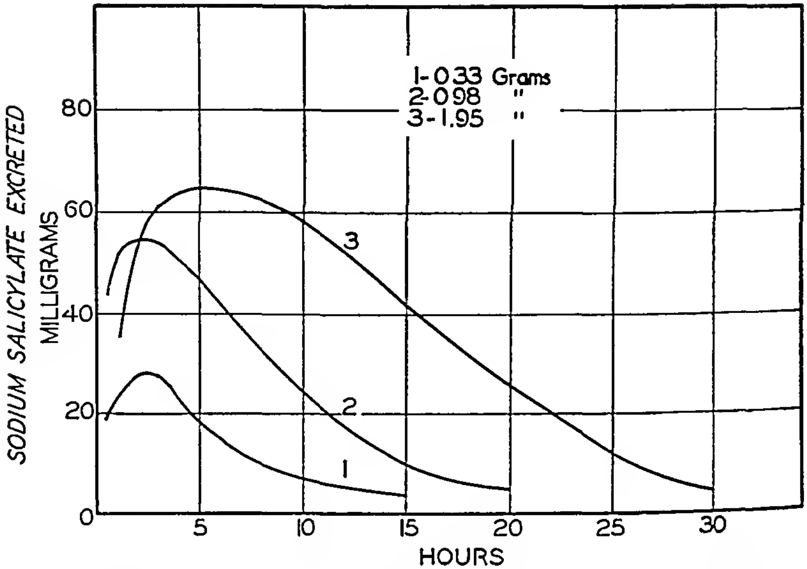

FIG 2 EXCRETION OF SALICYLATE (AS SODIUM SALICYLATE) PER HOUR AFTER ACETYLSALICYLIC ACID

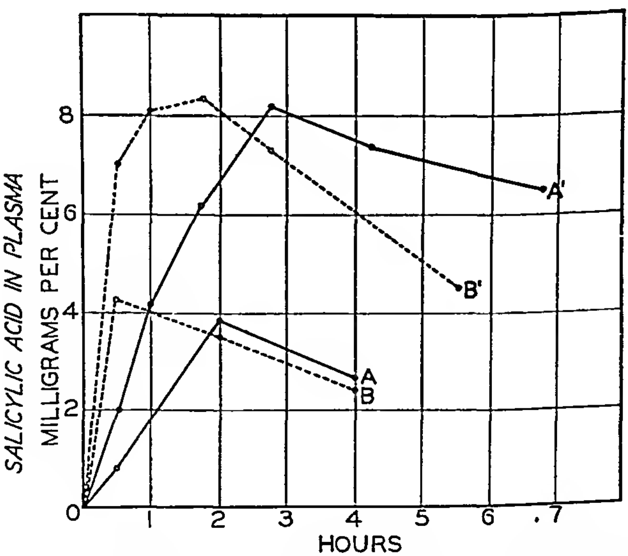

FIG 3 A, 0 65 g acetylsalicylic acid, A', 1 30 g acetylsalicylic acid; B, 0 65 g acetylsalicylic acid with 1 g $NaHCO_3$, B', 1 30 g acetylsalicylic acid with 2 g $NaHCO_3$

the observation that on incubation at 37° with plasma, the acetylsalicylate is hydrolyzed rapidly.

An attempt was made to determine the amount of the acetyl derivative in the plasma after oral administration; this was taken as the difference between total salicylate (by hydrolysis of plasma) and free salicylate. The probability that this difference represents acetylsalicylate rather than some other conjugated form is high for the following reasons: 1) After administration of sodium salicylate, no conjugated form could be detected in the plasma. 2) After administration of acetylsalicylic acid a conjugated form is present for a short time and in amounts varying with the dose given. 3) If this conjugated form occurred as a metabolite it would be anticipated after sodium salicylate as well as after acetylsalicylic acid and should be present in the plasma for the full time that salicylate was present instead of only for a short time after administration, as found here. 4) The possibility that the conjugated form, found after administration of acetylsalicylic acid, was salicyluric acid (o-hydroxyhippuric acid), an important urinary product of salicylates, was disproved by the absence of an increase in amino acids in the protein-free plasma filtrates after administration of salicylates. Determination of amino acids was made by the Russell (4) modification of the Frame, Russell and Wilhelmi (5) method. Brodie, Udenfriend and Coburn (1), employing a method using differential solvents, were unable to find salicyluric acid in plasma.

Thirty minutes after the ingestion of 0.65 g. of acetylsalicylic acid 27 per cent of the total salicylate present was acetylsalicylate; at 120 minutes, none could be found. Seventy minutes after the ingestion of 2.60 g., 13 per cent of the total salicylate was present as acetylsalicylate; at 160 minutes, none could be detected.

Effect of bicarbonate. Smull, Wégria and Leland (11) have reported that the administration of bicarbonate with sodium salicylate to both rheumatic and non-rheumatic subjects resulted in a lower concentration of salicylate in the plasma than when the salicylate was given without bicarbonate. They reported no measurement of urinary excretion. Goodman and Gilman (8) state that bicarbonate has no effect on the excretion of salicylates, and Hanzlik (9, 10), on one occasion, that it does not and, on another, that it does. Morris and Graham (12) reported an increase of salicylate in the urine of rheumatic patients given bicarbonate Table 1 shows the findings obtained with a normal subject receiving small amounts of salicylate After the administration of 2.60 g. of acetylsalicylic acid without bicarbonate, 925 mg. were eliminated in the urine in 12 hours; when the same amount was administered with 7.8 g. of sodium bicarbonate, 1501 mg were eliminated in 12 hours. This increase of more than 50 per cent in the rate of elimination is in excess of any individual variation in excretion found in any of the subjects studied. These findings are in agreement with others in which the excretion was followed for a shorter period; in all instances, bicarbonate increased the elimination of salicylate.

The effect on the concentration in the plasma, however, was not caused by increased elimination alone. As seen from figure 3, curves B and B′ in contrast

with curves A and A′, the concentration of salicylate in the plasma 2 or more hours after administration was lower when bicarbonate was given. With the bicarbonate, however, about the same maximum concentration was reached but at a much earlier period than when no bicarbonate was given. The bicarbonate markedly increased the rate of absorption, a finding which is in agreement with that of Schnedorf, Bradley and Ivy (13).

Accumulation. In experiments carried out here, four subjects took 1.95 g. of of acetylsalicylic acid at one time each day for 11 days. Analyses of the urine of the four subjects at the 35th and 36th hours after the last dose, showed less than 5 mg. present, indicating virtually complete elimination within this period of any amount accumulated. Since 30 hours are required for the complete elimination of a single dose of 1.95 g., this indicates that the amount of salicylate accumulated is insignificant.

From the values for excretion of salicylate in the urine obtained by Kapp and Coburn ((3) p. 352, fig. 10) and the values for concentration of salicylate in

TABLE 1

Subject taking 2.60 g. acetylsalicylic acid

TIME INTERVALS	URINE VOLUME	ACETYLSALICYLIC ACID EXCRETED
Without $NaHCO_3$		
hr.		*mg.*
0–6	620	645 } 925
6–12	490	280 }
With 7.8 g. $NaHCO_3$ in first 3.5 hr.		
0–6	425	886 } 1501
6–12	600	615 }

plasma obtained by Coburn (14) on repeated administration it is seen that the amount accumulated in the body is small, since after administration was stopped the amount present disappeared rapidly. Similar results were obtained by Butt, Leake, Solley, Griffith, Huntington and Montgomery (15).

THE BINDING OF SALICYLATE BY PLASMA. Jacoby (15), in 1908, was the first to point out that salicylate could be bound to serum constituents; he concluded from experiments on rabbits that salicylate is bound in some manner to the protein or polypeptides of serum. However, according to Jacoby, this binding could only be demonstrated in serum obtained after sodium salicylate administration *in vivo*; no binding seemed to result if the salicylate was added *in vitro* to serum. Jacoby offered no explanation for this difference and no investigators, including the present, have been able to reproduce it.

In 1923 Chabanier, Lebert, and Lobo-Onell (17) found that serum containing sodium salicylate lost practically none of the salicylate to isotonic saline, even after prolonged dialysis, while serum dialyzed against isotonic saline containing

salicylate rapidly gained more salicylate with its virtual disappearance from the saline. To determine if this same adsorption also obtained *in vivo* they measured the rate of urinary excretion of salicylate and the concentration in the serum, performing clearance measurements on subjects receiving salicylate. With no direct evidence they then made the assumption that the clearance of salicylate was identical with that of urea. On the basis of this assumption they concluded that the proportion of serum salicylate regulating excretion by the kidney is only a small part of the total salicylate held by the serum, and that the unbound fraction *in vivo* is greater than *in vitro*. Data to be presented here give experimental support to the assumptions and conclusions of Chabanier, Lebert and Lobo-Onell.

van Leeuwen and Drzimal (18) extended the *in vitro* dialysis observations to serum from asthmatics and reported that the capacity of this serum to bind salicylate was less than that of serum from normal individuals.

The binding of a drug to protein of the plasma should, on theoretical grounds, affect its excretion, distribution, and possibly its therapeutic efficiency. That distribution and excretion are affected has actually been shown for the sulfonamide group, the effect depending on the extent of the binding (19, 20, 21, 22). The effect on the therapeutic efficiency seems to depend on the particular drug (23, 24, 25, 26).

The present data on salicylate accord fully with the general statement of Reinhold, Flippin, Domm, and Pollack (22) that "sulfonamide so bound in a protein-sulfonamide complex appears to be in equilibrium with the free sulfonamide and plasma proteins. The penetration of sulfonamides into erythrocytes and other cells, and into cerebrospinal and other body fluids, including the glomerular filtrate, is confined to the ultrafiltrable fraction."

Methods. The two methods in general use for the determination of an unbound material in plasma or serum, dialysis and ultrafiltration, give similar results with salicylate. Dialysis has several disadvantages; it is slow, it is difficult to prevent dilution of the plasma by passage of water from buffer to plasma, and, for use in the determination of unbound salicylate *in vivo*, it has a fundamental disadvantage in that it lowers the original salicylate concentration of the plasma by diffusion into the dialysate. The important asset of dialysis is that the quantity of dialysate is suitable for accurate measurement, which is important for measuring unbound salicylates since the concentration is low and the analytical method of only moderate sensitivity.

For the determination of unbound salicylate, ultrafiltration does not, except for volume, suffer the disadvantages outlined for dialysis, because there is no significant alteration of the homogeneity of the plasma system. Two methods of ultrafiltration were tried. The first involved the use of a collodion covered sintered glass filter stick into which the plasma was admitted and 20 p.s.i. N_2 was applied for the filtration. Salicylate was variably retained upon the collodion depending on the preparation of the film, especially the rate and degree of evaporation of the solvent. If the solvent was removed completely, salicylate was not retained, but the film contracted and no longer adhered to the glass; plasma had therefore to be added to the stick and connection made to the nitrogen supply very rapidly in order to ensure a pressure-tight seal.

The second method which was tried worked more satisfactorily. The plasma to be analyzed was placed in a seamless cellophane bag made from tubing, suspended in a 50 cc. centrifuge tube and centrifuged at 2000 r.p.m. In preparation the two open ends of the

tubing were bent upward and 3 to 4 cc. of plasma admitted. Both openings were then closed by twisting and then knotting the cellophane. Strong cotton thread was wound and knotted below the cellophane knots and then used to suspend the bag in the centrifuge tube; this was facilitated by placing a hook just below the outside rim of the centrifuge tube. The tube was stoppered to prevent loss by evaporation. The yield of ultrafiltrate was about 0.25 to 0.30 cc. per hour. There was no determinable adsorption of the salicylate on the cellophane membrane and no protein was filtered.

The results obtained from dialysis and ultrafiltration were compared by dialyzing plasma containing salicylate against a buffer[3] until equilibrium was reached. The same equilibrium was reached by dialyzing plasma, which was free from salicylate, against a buffer containing salicylate. The plasma was then ultrafiltered and the concentration in the ultrafiltrate compared with that in the plasma water as calculated from the concentration in the buffer. In this way, disturbances caused by passage of water from dialysate into the plasma were eliminated. Agreement of the values was satisfactory within ±4 per cent.

Effect of concentration of salicylate. Both *in vivo* and *in vitro* plasma from different normal individuals and from the same individual at different times varied somewhat in its ability to bind salicylate. In general, the per cent of unbound salicylate increased with increase in concentration of salicylate. Some representative values are: a binding of 90 per cent at a total concentration of salicylic

TABLE 2

PROTEIN	BUFFER	PLASMA	SALICYLATE BOUND BY PLASMA	RATIO OF BOUND TO FREE SALICYLATE PER G. PROTEIN*
g. per cent	*mg. per cent salicylic acid*		*per cent*	*per cent*
5.45	1.33	6.86	81	77
2.80	1.61	6.27	74	104
1.92	1.68	5.34	69	114

* See Davis (23).

acid of 4 mg. per cent; 70 per cent at 16 mg. per cent; and 54 per cent at 70 mg. per cent. The binding capacity of plasma for salicylate is therefore high.

In rheumatic fever patients receiving salicylate therapy a much lower per cent of salicylate was bound to plasma than in normal individuals; in one instance, only 6 per cent was bound at a total salicylic acid concentration of 37 mg. per cent in plasma.

Effect of concentration of protein. Portions of the same plasma were suitably diluted with buffer and dialyzed against the buffer containing salicylate. The results are tabulated in table 2.

The binding, as seen, decreases with decrease in concentration of protein but the ratio increases and varies linearly with the concentration of protein. For the sulfonamides, Davis (23) found this ratio to decrease slightly with decreases in concentration of protein, although he found the overall percentage binding to be decreased, as in the present data on salicylate.

Effect of temperature. Three portions of the same sample of plasma were dialyzed for 20 hours at 6°, 20° and 37°C. against buffer containing the same initial

[3] The buffer used throughout the dialysis experiments was that employed by Davis (23), 0.15 M NaCl in 0.01 M phosphate buffer at pH 7.4.

concentration of salicylate. The results of one such series of determinations are given in table 3. Because the rate of diffusion increases with rise of temperature, the plasma at 37°C. is diluted the most. If correction is made to an equal concentration of protein in the three samples of plasma, it is found that temperature exerts no appreciable effect on the magnitude of the binding.

Binding of acetylsalicylate. The comparative binding of acetylsalicylate and salicylate was investigated by adding acetylsalicylate to the buffer and determining the concentrations of total and free salicylate in the plasma and buffer reached in 4 hours. The dialysis was carried out at 20°C. It was found that the binding power of plasma for free salicylate is much higher than for acetylsalicylate. Thus in a typical experiment in a series of such determinations, 92 per cent of the salicylate was bound to the plasma at a concentration of 5.8 mg. per cent salicylic acid while 33 per cent of the acetylsalicylate was bound at a total concentration of 12.0 mg. per cent. The binding of acetylsalicylate might

TABLE 3

TEMPERATURE	BUFFER	PLASMA	SALICYLATE BOUND BY PLASMA	DILUTION OF PLASMA
°C.	*mg per cent salicylic acid*		*per cent*	*per cent*
6	0.56	8.39	93 3	19
20	0.64	7.80	91.8	25
37	0.89	7.21	87.6	31

TABLE 4

Distribution of salicylate between plasma and red cells

Concentration as mg. per cent salicylic acid

WHOLE BLOOD	PLASMA	RED CELLS	RED CELL WATER	ULTRAFILTRATE OF PLASMA
38.3	52.0	20.7	27.6	27.1
30.0	42 2	14.5	19 3	18.6
20.3	28 8	9 4	12.5	11.3

be expected to increase somewhat with decrease in its total concentration, just as with the nonconjugated form; also, the binding might be arrested or reversed with the progressive decrease in acetylsalicylate and increase of salicylate as found to occur *in vivo*. These matters were not investigated since our point of interest bore only on the possibility, to be discussed later, that acetylsalicylate has a more potent analgesic action than salicylate, even though it occurs in low concentrations in the plasma after administration. If, for this effect, only unbound free salicylate and unbound acetylsalicylate were effective the unequal binding of salicylate and acetylsalicylate, as shown here, would proportionally reinforce the effectiveness of the acetylsalicylate.

THE DISTRIBUTION OF SALICYLATES IN BODY FLUIDS. *Between plasma and red cells.* The distribution of salicylate between plasma and red cells was obtained by adding sodium salicylate to blood and determining the concentration of

salicylate in the whole blood, plasma, and ultrafiltrate of the plasma. The concentration of salicylate in the red cells was calculated from the hematocrit volume and the concentration of salicylate in whole blood and plasma, an indirect determination which is subject to some error (27). The data obtained are given in table 4. As seen there, the concentration of salicylate in the water of the red cell (concentration of total salicylate in the red cells divided by the percentage of water in the red cells) is, within the range of error of determination, identical with that of the unbound salicylate in the plasma water. There is, therefore, no binding of salicylate by the protein of the red cell. This proof was again obtained by suspending saline washed red cells in saline to which sodium salicylate was added and determining the concentrations of salicylate present in the water of the red cell and in the saline; these concentrations were again equal. The data presented here are in contradistinction to those of Coburn (14) who states that the content of salicylate in the red cell is "negligible."

Between plasma and cerebrospinal fluid. Because the experimental period was no longer than 8 hours, the results obtained in the present work indicate only that the equilibrium concentration of salicylate in cerebrospinal fluid probably approaches the concentration in ultrafiltrate of plasma and that the diffusion of

TABLE 5

TIME	WHOLE BLOOD*	PLASMA*	RED CELL WATER*	CEREBROSPINAL FLUID*
hr.				
5	34.2	41.4	34.2	13.0
8	35.8	44.7	33.7	16.2

* In mg. per cent salicylic acid.

unbound salicylate into the cerebrospinal fluid, and the reverse process, are slow, requiring many hours for the attainment of a steady state.

A dog weighing 7 kg. was put under partial anesthesia with sodium amytal and given 200 mg. per kg. of sodium salicylate intravenously; 1 hour later an infusion of sodium salicylate solution was begun into the right jugular vein at the rate of 20 mg. of salicylate per kg. per hour (12 cc. solution per hour) and continued for 7 hours. At 5 and 8 hours after the initial administration of salicylate, samples of blood and cisternal fluid were withdrawn. The cisternal fluid contained the normal small amount of protein, of such magnitude that binding of salicylate did not have to be considered. The data obtained are given in table 5. Since it was found that for all practical purposes the concentration of salicylate in the water of the red cell is equivalent to that of the ultrafiltrate of plasma, the former was used as a measure of the latter quantity.

Another dog, weighing 7.6 kg., was given, under ether anesthesia, 200 mg. per kg. of acetylsalicylic acid[4] intravenously. At 1 hour and $5\frac{1}{4}$ hours blood and cisternal fluid samples were drawn, with the findings shown in table 6.

[4] The acid was brought into solution (5.6 per cent) with sodium hydroxide. The solution was slightly acid when given and contained 8 per cent salicylic acid.

The fact that acetylsalicylate appears in the cisternal fluid when none is present in the blood would indicate that it is not hydrolyzed as rapidly in this medium as in the blood.

Between plasma and peritoneal fluid. The difficulty in obtaining more than infinitesimal amounts of peritoneal fluid under normal circumstances made it necessary to utilize the technique of Schechter (28), which consists of increasing the volume of peritoneal fluid artificially by the injection of saline into the peritoneal cavity.

Two rats were given 200 mg. per kg. of sodium salicylate intravenously and 20 cc. of saline intraperitoneally. At 2 hours blood was drawn by cardiac puncture from one rat, the animal killed by an air embolus, an incision made into the belly wall and the peritoneal saline withdrawn. At $5\frac{1}{6}$ hours blood and peritoneal saline were obtained similarly from the second rat. Table 7 gives the data obtained. The agreement between the concentration of salicylate in the

TABLE 6

TIME	WHOLE BLOOD		PLASMA		RED CELL WATER		CISTERNAL FLUID	
	(a)*	(b)	(a)	(b)	(a)	(b)	(a)	(b)
hr.	*mg. per cent salicylic acid*							
1	25.7	0	34.8	0	21.6	0	4.0	1.0
$5\frac{1}{4}$	15.6		22.3		9.6		6.6	

* (a) Salicylic acid, (b) acetylsalicylic acid.

TABLE 7

TIME	WHOLE BLOOD	PLASMA	RED CELL WATER	PERITONEAL SALINE
hr.	*mg. per cent salicylic acid*			
2	23.3	28.6	15.9	16.4
$5\frac{1}{6}$	18.6	21.1	14.2	11.5

water of the red cell and the peritoneal saline was satisfactory, but the possibility existed that equilibrium might not have been established in the time periods employed. In order to avoid a prolonged period the same experiment was repeated except that 1 hour after the injection of the sodium salicylate, saline containing 30 mg. per cent of sodium salicylate was injected intraperitoneally; this concentration was higher than the unbound salicylate in the plasma. Two hours after the injection of saline, blood and peritoneal saline were withdrawn. On analysis the concentration of salicylate (in mg. per cent salicylic acid) was found to be: whole blood, 28.3; plasma, 36.0; red cell water, 19.6; and peritoneal saline, 19.2.

This experiment, taken in conjunction with the two previous ones, provides strong evidence that at equilbrium salicylate is distributed throughout the non-protein extracellular water in a concentration equal to its concentration in the water of the red cell, and therefore that of the ultrafiltrate of plasma.

Discussion. From the distribution between plasma and red cells, cerebros-

pinal fluid, and peritoneal saline, there is no doubt that the equilibrium concentration of salicylate in these fluids is that of the ultrafiltrate of plasma. It is therefore logical to assume that the remainder of the extracellular water would also have at least this concentration. Penetration of the red cell by salicylate is no evidence of its distribution throughout the intracellular water. And since the portions of the extracellular water not investigated here may contain as much as 4 per cent of protein (29), it is impossible to reach a decision from the data here as to whether the salicylate is distributed throughout the whole of the body water or confined to the extracellular water.

Renal excretion. In the kidney, salicylates are in part conjugated to form salicyluric acid and possibly glucuronides. Since, however, it has been impossible to detect any endogenous conjugates in the circulating blood, the total salicylate present in the urine may be taken as the value used for calculating the clearance. Table 8 gives some representative clearance values found at different levels of salicylate in plasma.

TABLE 8

Renal excretion of salicylate

SUBJECT	DOSE	URINARY VOLUME	TOTAL SALICYLIC ACID IN PLASMA	UNBOUND SALICYLIC ACID IN PLASMA	CLEARANCE	
					Urea	Salicylate
	g.	cc./min.	mg. %	mg. %	cc./min.	cc./min.
1	1.63*	1.95	9.0	1.55‡	58	56
1	1.63*	7.00	8.7	1.40‡	63	63
1	1.95*	2.00	8.1	1.21§	79	99
2	0.98*	1.30	5.7	0.89‡	65	46
3	8.00†	2.33	17.7	9.50‡	71	71
3	10.00†	1.33	12.5	4.30‡	80	75

* Acetylsalicylic acid.
† Sodium salicylate to rheumatic patient.
‡ From ultrafiltrate of plasma.
§ From concentration in water of red cell.

With the exception of the third experiment on Subject 1, the clearances indicate that salicylate is excreted by glomerular filtration and tubular reabsorption, similar and practically equal to that of urea. In the third experiment an absolute increase of only a few tenths of a milligram per cent in the concentration of unbound salicylate would decrease the clearance from 99 cc. per minute, as recorded, to 79 cc. per minute, corresponding to the clearance of urea. A small absolute change in the concentration of unbound salicylate causes a large change in clearance; the possibility of error in determining the unbound salicylate is greatly enhanced if the determination of this concentration is indirect, as in the third experiment on Subject 1, and at low total concentrations in blood and plasma.

The means by which the urinary excretion is increased by bicarbonate have not been studied, but from the work presented here two processes suggest them-

selves: 1) an increase in the unbound salicylate fraction in plasma; and/or 2) a decrease in the tubular reabsorption; both processes would lead to an increase in the excretion of salicylate which would be reflected in an increased clearance rate in the latter case.

ANALGESIA. Clinical observations indicate that the analgesia afforded by acetylsalicylic acid lasts from 1 to 4 hours, depending somewhat on the dosage. The concentration of total salicylate in plasma persists many hours beyond this time. The short duration of the analgesia corresponds to the shorter period of time during which acetylsalicylate is present in the plasma. In addition to this coincidence in time is the fact that acetylsalicylic acid usually is considered a more effective analgesic agent than sodium salicylate. Acetylsalicylate, therefore, may, in ordinary therapeutic doses, be the effective analgesic agent. To obtain relief from rheumatic pain with sodium salicylate, the concentration of salicylate must be greater than 20 mg. per cent, that is, when the concentration of unbound salicylate becomes appreciable. With doses of 1 or 2 g. or less, the concentration of salicylate not bound to plasma may be below that necessary for analgesic action, whereas these doses of acetylsalicylic acid, by furnishing acetylsalicylate to the plasma, may raise the concentration to a therapeutically effective level.

If this hypothesis regarding the effective analgesic agent has validity, the simultaneous administration of bicarbonate would have a beneficent effect in hastening absorption and therefore speeding the analgesia. Since the duration of analgesia is relatively short, a more rapid excretion of the salicylate caused by the bicarbonate would not be disadvantageous.

CONCLUSIONS

The absorption, distribution and excretion of salicylate after doses of acetylsalicylic acid in amounts up to 1.95 g. have been studied.

From 52 to 75 per cent of the acetylsalicylic acid ingested is excreted in the urine in various forms. The time required for excretion ranges from some 15 hours for doses of 0.33 g. to some 30 hours for 1.95 g.

The maximum concentration of total salicylate reached in the plasma is of the order of 4 mg. per cent after doses of 0.65 g. and it varies approximately linearly with dosage. The administration of bicarbonate with acetylsalicylic acid increases the rate of absorption with a much earlier maximum concentration in the plasma but at about the same level; the bicarbonate also increases the rate of elimination of salicylate and hence the decrease in concentration after the maximum.

Acetylsalicylic acid is rapidly hydrolyzed after absorption but up to a period of 1 to 2 hours as much as a quarter of the salicylate in the plasma may be in acetylated form.

The accumulation of salicylate on repeated doses of acetylsalicylate is small.

The protein of the plasma exerts a strong binding action on salicylate, but less on acetylsalicylic acid.

The distribution of salicylate between the plasma and red cell and the peri-

toneal saline and cerebrospinal fluid is determined by the unbound fraction in plasma water.

The same fraction establishes the rate of elimination through the kidney. The clearance is similar and practically equal to that of urea.

The theory is advanced that the analgesic action of acetylsalicylate is exercised mainly by the unhydrolyzed acetylated fraction in the plasma.

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CHANGES IN THE LEUCOCTYE PICTURE IN EXPERIMENTAL TRYPANOSOMIASIS BY ADMINISTRATION OF NEOSTIBOSAN AND NEOSTAM[1]

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Fresh interest has been aroused in the study of the cellular response of the body to parasitic infections, because of the increasing recognition of the part played by the reticulo-endothelial system in the defense mechanism of the body against infections. Taliaferro (1) has shown that in protozoal disease, like malaria there is strong presumptive evidence for the belief that phagocytosis and destruction of the parasites by the cells of the reticulo-endothelial system constitute the chief mechanism of defense of the body. In trypanosome infection, in particular, it has been shown by Taliaferro (1) that the reticulo-endothelial system is not only responsible for the formation of humoral antibodies which inhibit cell division of the parasites, but it also furnishes cells for the engulfing and digestion of these parasites.

Gowe (2) has shown that in rabbits resistant to infection with *Trypanosoma equiperdum*, there was a significant increase in the relative per-centage and total number of monocytic cells of the body prior to crisis. On the other hand, non-resistant rabbits and rats showed no such increase and promptly succumbed to infection, in spite of the very marked increase in relative and absolute numbers of polymorphonuclear leucocytes. Poindexter (3) observed that from the first to the fourth week of infection with *Trypanosoma lewisi*, rats showed from 16 to 35 per cent of large monocytes. This increase in monocytes is believed to be responsible for the resistance of rats to *T. lewisi* infection.

Most studies hitherto with antimonials in trypanosomiasis have been concerned solely with their therapeutic effect. Literature concerning the effects of these drugs on the blood and hematopoietic organs during the course of infection is lacking. Zia and Forkner (4) gave daily intravenous injections of neostibosan to normal rabbits (total doses 2.40 to 4.79 mgm.) and did not observe an appreciable change in the white blood corpuscles of the test animals. Pan (5) gave repeated injections of lopin and neostibosan to normal rabbits and observed no appreciable hematological changes

The present study was undertaken to determine the effects of two antimony compounds on the blood in experimental trypanosomiasis. For this purpose, neostibosan (metachlor-paracetylaminophenyl stibiate of sodium) and neostam (nitrogen glucoside of paraminophenyl stibinate of sodium) were chosen because of their wide applicability as therapeutic agents for kala azar, their wide try-

[1] A further consideration of this investigation in relation to data on the leucocyte picture in experimental trypanosomiasis after adrenalin injection will be presented in a subsequent paper by the authors

panocidal activity and our interest in these drugs through studies of other pharmacological properties.

METHOD. Four groups of rats were used in these experiments. The first group consisted of five normal rats. The second group consisted of five rats infected with *Trypanosoma equiperdum*. The third and fourth groups of rats consisted of ten rats infected with *T. equiperdum* and treated with neostibosan and neostam respectively. The method of treatment consisted of injecting into the muscles

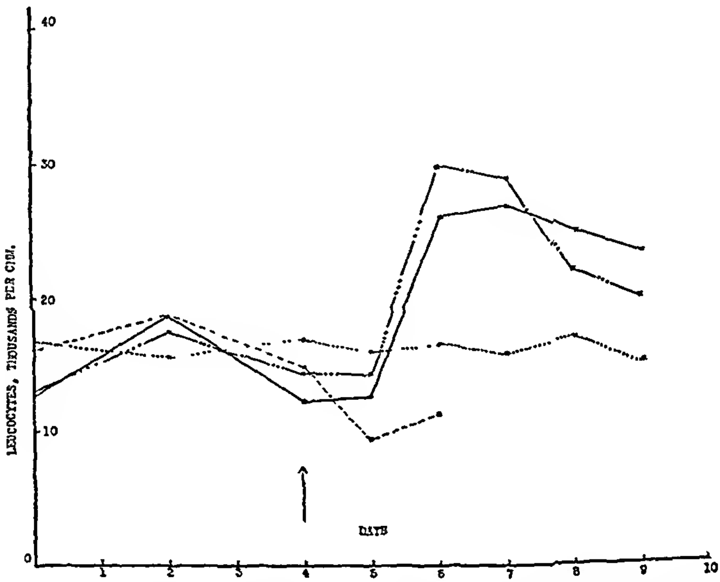

FIG. 1. TOTAL LEUCOCYTE CHANGES IN RATS TREATED WITH NEOSTIBOSAN AND NEOSTAM, UNTREATED AND NORMAL RATS

———, *T. equiperdum* infection treated with neostibosan.
— ... —, *T. equiperdum* infection treated with neostam.
------, *T. equiperdum* infection without treatment.
. . . . , normal rats.

The drugs were administered on the 4th day after infection (see arrow in this figure). 50 mgm. of drug given intramuscularly. Total and differential leucocyte counts were made prior to the administration of the drugs on that day. The animals were inoculated with trypanosomes on 0-day. Total and differential leucocyte counts were made before inoculation.

of the thigh 50 mgs. of neostibosan or neostam at the height of infection, generally four days after infection. In view of the interesting results of Zia and Forkner (4) and Pan (5) we considered superfluous the running of another control series with administration of neostibosan and neostam to normal rats.

The degree of infection was determined daily by the examination of the blood of the animals. A drop of tail blood was placed upon a slide and covered with a coverslip. This preparation was examined under the oil immersion objective of

the microscope. Examinations of each blood were made prior to infection and at twenty-four hour intervals after infection and administration of the drugs until the animals died or recovered from the infection. The same procedure was employed in the examination of blood from the normal rats. The blood was obtained from the rat's tails. The blood examinations consisted of total white blood cell and differential counts of the leucocytes. Leucocyte counts were made on blood diluted in certified Neubauer white pipettes using two per cent acetic acid to which were added a few crystals of gentian violet as diluting fluid. Each count was repeated twice to insure accuracy. Dried blood films were prepared

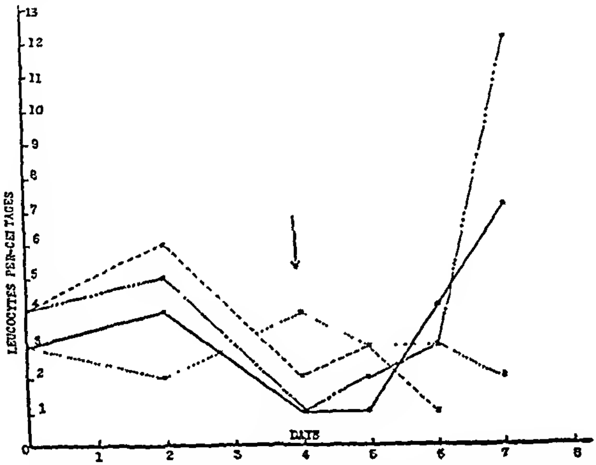

FIG. 2. MONOCYTIC CHANGES IN RATS TREATED WITH NEOSTIBOSAN AND NEOSTAM, UNTREATED AND NORMAL RATS

T equiperdum infection treated with neostibosan.
, *T equiperdum* infection treated with neostam.
– – –, *T equiperdum* infection without treatment.
, normal rats

The drugs were administered on the 4th day after infection (see arrow in this figure). 50 mgm. of drug given intramuscularly. Total and differential leucocyte counts were made prior to the administration of the drugs on that day The animals were inoculated with trypanosomes on 0-day Total and differential leucocyte counts were made before inoculation.

on slides for the differential counts and stained by modified Wright's stain (6). Duplicate counts of 100 cells each were made and the average recorded. Neutrophils, lymphocytes, monocytes, basophils and eosinophils were differentiated. The animals were conditioned by repeated handling before the experiments were started in order to avoid "emotional struggle" which is know to produce leucocytosis in rats.

Results. The results of the experiments are summarized in figures 1, 2, 3, and 4. Figure 1 gives the data of the total leucocyte counts in four groups of rats. Figure 2 gives the monocyte counts, figure 3 the neutrophil counts and figure 4 the lymphocyte counts, in per-centages.

The total leucocyte counts: The results of the total leucocyte counts show that in normal rats the variations from day to day under conditions of our experiments were not very significant. It is evident, however, that counts from individual rats varied from the average, but the individual variations were not statistically significant. Rats infected with *T. equiperdum* showed diminution in the number of total leucocytes. This was evident from the second day after infection. The average life of rats was six days. Those rats whose blood was examined at the terminal stage of infection showed premortal leucocytosis. Such counts

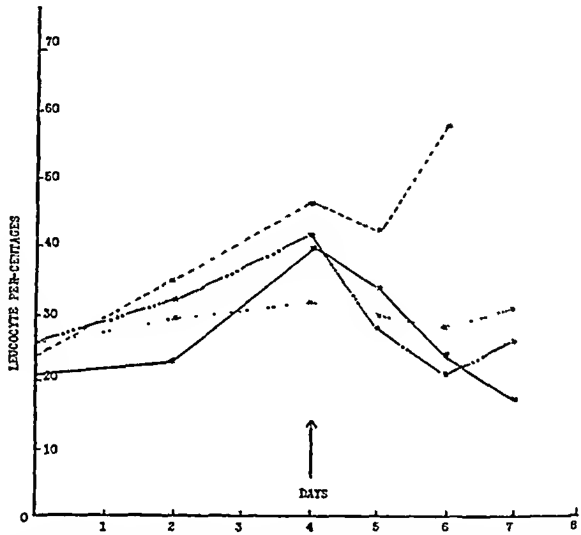

FIG. 3. POLYMORPHONUCLEAR NEUTROPHILIC CHANGES IN RATS TREATED WITH NEOSTIBOSAN AND NEOSTAM, UNTREATED AND NORMAL RATS

———, *T. equiperdum* infection treated with neostibosan.
— . —, *T. equiperdum* infection treated with neostam.
- - - - - -, *T. equiperdum* infection without treatment.
. . , normal rats.

The drugs were administered on the 4th day after infection (see arrow in this figure). 50 mgm. of drug given intramuscularly. Total and differential leucocyte counts were made prior to the administration of the drugs on that day. The animals were inoculated with trypanosomes on 0-day. Total and differential leucocyte counts were made before inoculation.

were discarded. Both groups of rats infected with *T. equiperdum* and treated with neostibosan or neostam four days after infection, showed marked leucocytosis. This was not evident until twenty-four hours after the administration of the drugs.

The differential counts: The lymphocytes, polymorphonuclear neutrophils, monocytes, basophils and eosinophils did not show significant variations from day to day determinations in normal rats. Our results are in agreement with the observations of other investigators (7). In rats infected with *T. equiperdum*,

the differential counts showed a decrease in lymphocyte per-centage and increased polymorphonuclear neutrophil per-centage. These results are in agreement with the observations of Gowe (2). There was a significant decrease in monocyte per-centage. In rats infected with *T. equiperdum* and treated with neostibosan or neostam, the differential counts reveal that prior to treatment, after the counts were taken on the fourth day after infection, there was a decrease in lymphocyte per-centage, an increase in polymorphonuclear neutrophil per-centage

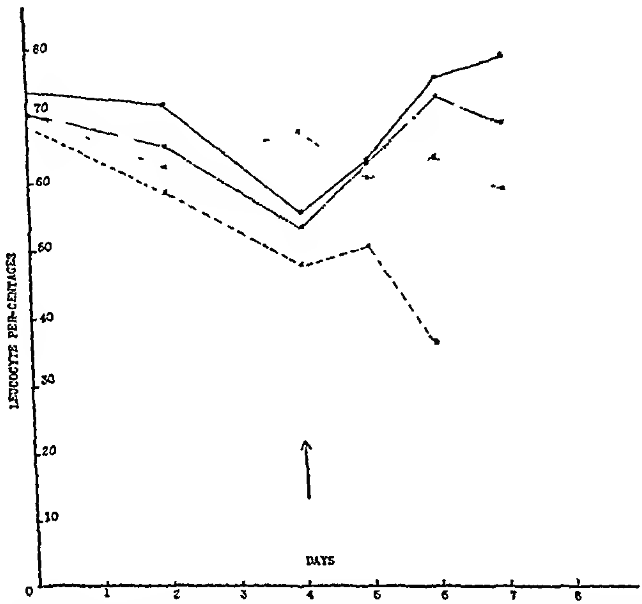

FIG 4 LYMPHOCYTE CHANGES IN RATS TREATED WITH NEOSTIBOSAN AND NEOSTAM, UNTREATED AND NORMAL RATS

-, *T equiperdum* infection treated with neostibosan.
–, *T equiperdum* infection treated with neostam.
- –, *T. equiperdum* infection without treatment
, normal rats

The drugs were administered on the 4th day after infection (see arrow in this figure). 50 mgm of drug given intramuscularly Total and differential leucocyte counts were made prior to the administration of the drugs on that day. The animals were inoculated with trypanosomes on 0 day Total and differential leucocyte counts were made before inoculation.

and a decrease in monocyte per-centage. Following treatment, however, the lymphocyte per-centage increased with a decrease in polymorphonuclear neutrophil per-centage while the monocytes increased. The increase in the monocytes was more than 100 per cent.

The changes in the basophils and eosinophils, in all cases, were not very significant.

Discussion It will be seen from figures 1, 2, 3 and 4 that the curves for neo-

stibosan and neostam follow similar trends. There was an appreciable change in all cases, in total leucocyte as well as differential counts, after the administration of the drugs. It is obvious from our results that as a result of trypanosome infection in rats without any manifestation of resistance, the animals were unable to effect a crisis. The monocyte per centage decreased and was abnormally low until death. On the other hand, however, in those animals treated neostibosan or neostam, the depleted monocytes greatly increased twenty-four hours after the administration of the drugs.

Taliaferro (8) and Linton (9) have discussed and reviewed the importance of the reticulo-endothelial system in trypanosome infections. Gowe (2) and Poindexter (3) have shown that an increase in the number of monocytic cells occur after infection and may help in antibody production in animals which do not succumb to infection. No evidence has been produced to show that monocytosis is the only basis of resistance to trypanosome infection.

Chemotherapeutic agents as arsenicals and germanin (Bayer 205) have been shown to depend for their action on the co-operative function of the reticulo-endothelial cells (Jungeblut (10); Reiner, Leonard, and Chao (11); von Jansco and von Jansco (12); and Voegtlin and Thompson (13); Culbertson (14)). When these cells are blockaded with India ink or when an animal is splenectomized and thereby deprived of a large and rich supply of the reticulo-endothelial tissue, specific arsenical or germanin suffer severe impairment of function. To these drugs, neostibosan and neostam (antimonials) can now be added. Our present information, from the results of our experiments seems to show that the monocytes are scavengers and play an adjunctive role by completing the destruction of trypanosomes after they have been rendered vulnerable by the drugs.

SUMMARY

1. In rats which show no resistance to infection with *Trypanosoma equiperdum*, the most characteristic change in the leucocyte picture is a decrease in lymphocyte per-centage, increase in polymorphonuclear neutrophil per-centage and a decrease in monocyte per-centage.

2. Rats infected with *T. equiperdum* and treated with neostibosan or neostam showed increased monocyte per-centage, decreased polymorphonuclear neutrophil per-centage and increased lymphocytes, after treatment.

3. It is suggested that the increased monocytes play adjunctive role in respect to the drugs (neostibosan and neostam) by completing the destruction of trypanosomes after they have been rendered vulnerable by the drugs.

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University of Toronto, and Miss Norah Butterworth, St. Joseph's Hospital, Toronto, for their technical assistance.

The neostibosan was generously provided by the Winthrop Chemical Company of New York City. The authors wish to thank Dr. J. B. Price, Director of the Research Division of this company for his interest in this work. The neostam was kindly supplied by the Wellcome Research Laboratories, Tuckahoe, New York.

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THE PROTECTIVE ACTION OF ATABRINE AGAINST CHLOROFORM-ADRENALINE VENTRICULAR FIBRILLATION

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It has been previously reported from this laboratory (1) that ventricular fibrillation, following injection of a large dose of posterior pituitary extract in the phenobarbitalized dog, can be prevented by previous administration of various coronary dilator agents. It was suggested that this effect was due mainly to a partial or complete prevention of the coronary constrictor action of the extractor.

During the course of recent experiments concerning the effects of atabrine on the heart, it was observed by Babkin and the writer (2) that atabrine induces marked coronary dilatation in the isolated perfused rabbit heart. It was also observed that atabrine induces a partial or complete paralysis of the cardio-inhibitory vagal response to electrical stimulation in dogs under pentobarbital anaesthesia.

In view of the above findings it was of interest to test the effects of atabrine on ventricular fibrillation induced by different procedures. The object of this paper is to present the results which were obtained from such an investigation in connection with chloroform-adrenaline ventricular fibrillation.

Methods. Dogs anaesthetized with pentobarbital sodium (nembutal) 40 mgm. per kgm., were used. A tracheal cannula was inserted, and artificial respiration maintained throughout each experiment, using a Starling pump.

The administration of chloroform was carried out by connecting the chloroform bottle with the inlet of the artificial respiration circuit (close to the tracheal cannula). The quantity of chloroform administered was adjusted so that there was only a slight fall in blood pressure. After 5 minutes of administration a dose of 0.02 mgm. per kgm. of adrenaline in 1 cc. of distilled water was injected intravenously and the chloroform discontinued one minute later in most experiments. Such a procedure regularly induced ventricular fibrillation in the pentobarbitalized dog.

The influence of atabrine was tested either (a) by making a quick single injection in the usual manner just prior to or during the chloroform administration, or (b) by slow prolonged intravenous infusion of the solution at a fixed rate of 5 mgm. per cc. per minute. The latter procedure had the advantage that much larger quantities of the drug could be employed without any apparent deleterious effect on blood pressure, which invariably showed a fall when the more rapid administration of the drug was employed. A 0.5% solution of atabrine hydrochloride in physiological saline was used in all experiments.

The blood pressure was recorded directly from a common carotid artery, and electrocardiograms (Lead II) taken at frequent intervals during the course of several experiments.

The influence of vagotomy or atropinization upon the chloroform-adrenaline response after atabrine was also similarly studied.

All injections were made into an exposed femoral vein.

Results. In table 1 are summarized results of several experiments in which the influence of a single injection of atabrine upon chloroform-adrenaline

ventricular fibrillation was tested. As can be seen, in expts. 1, 2, 3 and 4, when no atabrine or an insufficient dose was employed, ventricular fibrillation occurred in all four experiments following the first chloroform-adrenaline administration.

TABLE 1

Influence of a single injection of atabrine upon chloroform-adrenaline ventricular fibrillation in dogs

EXPT. NO.	WT.	DOSE OF ATABRINE	TIME OF INJECTION IN RESPECT TO CHLOROFORM	EFFECT OF FIRST CHLOROFORM-ADRENALINE*	EFFECT OF SUBSEQUENT REPETITIONS OF CHLOROFORM ADRENALINE
	kgm.	*mgm /kgm.*			
1	19.7	none		fibrillation	
2	10.0	none		fibrillation	
3	7.0	1.0	2 min. after start	fibrillation	
4	4.4	2.5	2 min. after start	fibrillation	
5	7.2	5.0	1 min. after start	no fibrillation	20 min. later fibrillation
6	7.0	5.0	4 min. after start	no fibrillation	15 min. later fibrillation
7	8.3	5 0	1 min before start	no fibrillation	10 min. later fibrillation
8	11.3	5.0	2 min. before start	no fibrillation	10 min. later fibrillation
9	6.4	5.0	3 min. before start	no fibrillation†	10 min. later fibrillation
10	5.0	5.0	5 min. before start	fibrillation	

* 5 minutes of chloroform followed by injection of adrenaline (.02 mgm./kgm.)

† Adrenaline was injected after 3 minutes of chloroform in this experiment (see fig. 1)

TABLE 2

Influence of prolonged intravenous administration of atabrine upon chloroform-adrenaline ventricular fibrillation in dogs

EXPT. NO.	WT.	TOTAL DOSE OF ATABRINE	TIME OF INJECTION	EFFECT OF FIRST CHLOROFORM-ADRENALINE*	EFFECT OF SUBSEQUENT REPETITIONS OF CHLOROFORM-ADRENALINE
	kgm.	*mgm /kgm*	*min*		
1	11.6	2.10	5	fibrillation	
2	8.7	2.87	5	fibrillation	
3	13.7	9.90	27	no fibrillation	10 min. later fibrillation
4	11.9	11.34	27	no fibrillation	10 min. later fibrillation
5	10.6	12.26	26	no fibrillation	(a)10 min. later no fibrillation (b)10 min. later fibrillation
6	12.1	12.40	30	no fibrillation	15 min. later fibrillation
7	12.6	19.70	50	no fibrillation†	10 min. later fibrillation

* 5 minutes of chloroform and adrenaline (.02 mgm /kgm) were administered just before the end of the atabrine injection.

† In this experiment, two unsuccessful attempts were made during the atabrine injection (see fig. 2).

On the contrary, in expts. 5, 6, 7, 8 and 9 when a dose of 5 mgm. per kgm. of atabrine was injected intravenously either shortly before (1 or 2 minutes) or during the chloroform administration, a similar injection of adrenaline induced no fibrillation. Furthermore, in each of these experiments, when the chloroform-

adrenaline administration was repeated after an interval of 10 to 20 minutes ventricular fibrillation occurred in every case. The effect of atabrine is therefore rather transitory, and indeed, in experiment 10, when a similar dose of atabrine was given 5 minutes before the chloroform was started, i.e., 10 minutes before the adrenaline injection, there was no evidence of protection and fibrillation ensued.

In view of the transitory effects of atabrine in the above experiments, we were curious as to whether or not this protection could be prolonged by slow continuous administration of larger doses of the drug. In table 2, are summarized results of several such experiments. Here again experiments 1 and 2 show that with insufficient dosages of atabrine ventricular fibrillation ensued upon the first chloroform-adrenaline administration. On the contrary, in experiments 3, 4, 5, 6, and 7, when total doses of 9.9 to 19.7 mgm. per kgm. of atabrine were injected at a slow rate, i.e., 5 mgm. of atabrine per minute, there was no fibrillation with chloroform and adrenaline, but again after 10 to 20 minutes following the cessation of the atabrine infusion, the administration of chloroform and adrenaline as before induced fibrillation in every instance. There was therefore no significant prolongation of the protective action of atabrine, despite the high dosages employed. More will be said about this later.

In figures 1 and 2 are reproduced examples of the blood pressure changes which were observed in these two groups of experiments. These figures are self-explanatory, and need no further comment.

More or less similar results were obtained in several other experiments of the same types in which the animals were previously double vagotomized or atropinized.

It should also be added that all attempts to restore the fibrillating ventricle by intracardiac injections of atabrine and subsequently massaging the heart for several minutes, were unsuccessful. It was nevertheless possible to protect the heart against fibrillation by injecting atabrine and adrenaline at the same time. Thus, in figure 3, are shown results of two such experiments, in which when a dose of 2.5 mgm. per kgm. of atabrine was mixed with 0.02 mgm. per kgm. of adrenaline, and the mixture injected (A and A1) during chloroform administration, there was no evidence of ventricular fibrillation. There was a prompt rise in blood pressure in each instance.

Electrocardiographic Observations. In view of the intense adrenaline effects resulting from the large doses employed in the above experiments, and their possible complicating influence upon the electrocardiograms, it was thought that a study of the effects induced by smaller doses might simplify the electrocardiographic alterations and perhaps throw some light upon the mechanism of this protective action of atabrine. In figures 4, 5, 6 and 7 are shown results from two such experiments. In each of these a dose of .002 mgm. per kgm. of adrenaline was employed. It should be stated that such a dose of adrenaline was often quite sufficient to induce ventricular fibrillation during chloroform although this did not occur in every instance. The associated blood pressure changes are also shown in each of these experiments.

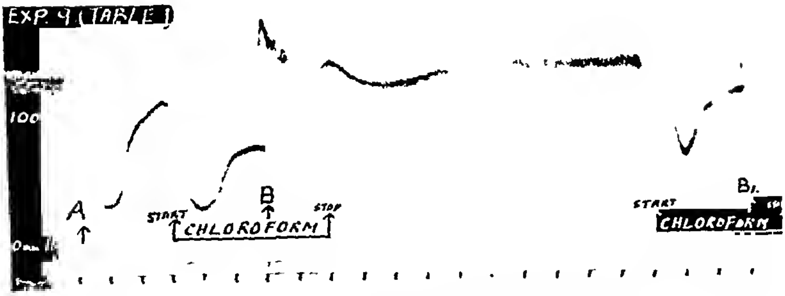

FIG. 1. Dog, female, 6.4 kgm Sodium pentobarbital anesthesia. Artificial respiration. Blood pressure tracing. A—atabrine hydrochloride (5 mgm. per kgm.). B and B1—adrenaline (.02 mgm. per kgm.)

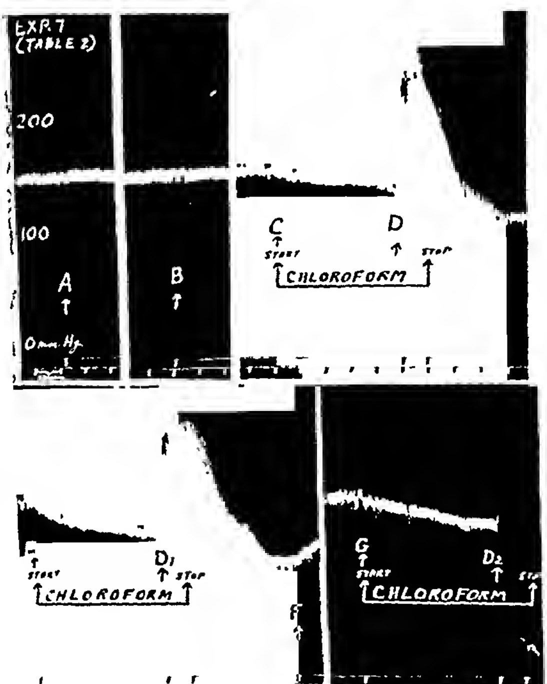

FIG 2 Dog, male, 12 6 kgm Sodium pentobarbital anesthesia Artificial respiration. Blood pressure tracing From A to F (50 minutes) atabrine hydrochloride (5 mgm per cc. per min) was injected—B, C, and E—10, 20, and 10 minutes respectively, after atabrine was started D, D1 and D2—adrenaline (02 mgm per kgm) An interval of 15 minutes elapsed between D and E and an interval of 10 minutes between D1 and G

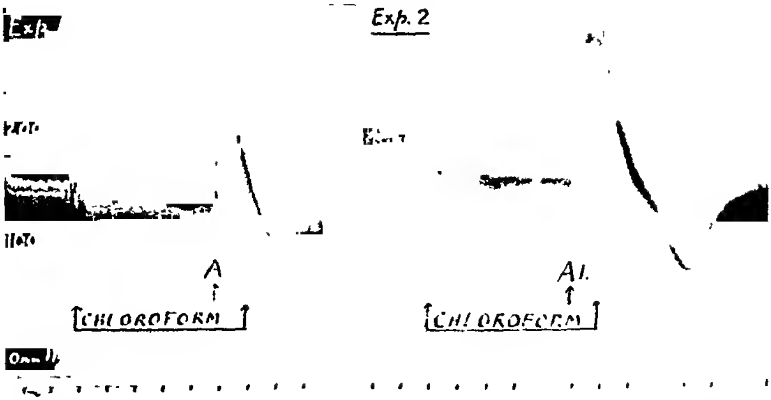

Fig. 3. *Expt. 1.* Dog, male, 5.3 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Blood pressure tracing. At A, a mixture of 2.5 mgm. per kgm. of atabrine hydrochloride and 0.02 mgm. per kgm. of adrenaline, was injected.

Expt. 2. Dog, female, 4.75 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Blood pressure tracing. At A, a mixture of 2.5 mgm. per kgm. of atabrine hydrochloride and 0.02 mgm. per kgm. of adrenaline was injected.

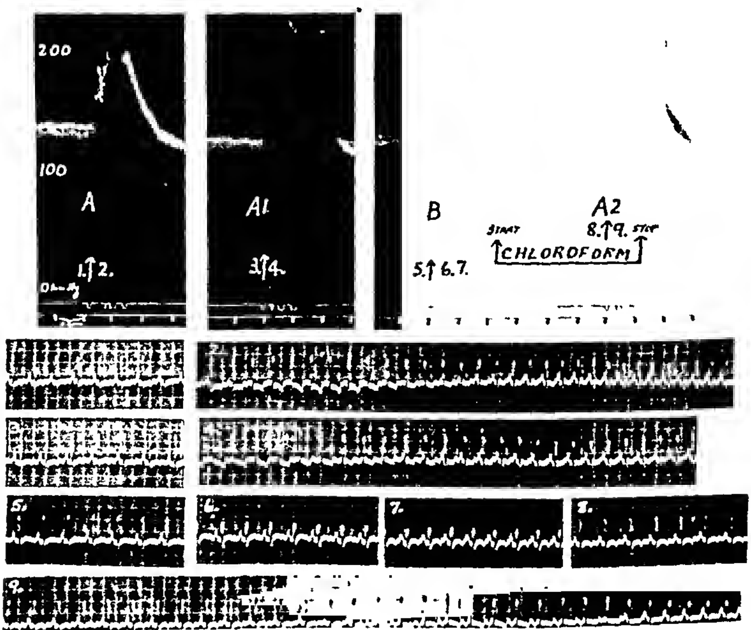

Fig. 4. Dog, male, 11.8 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Blood pressure tracing and electrocardiograms (Lead II). At A, A1, and A2, adrenaline (.002 mgm. per kgm.) was injected, and at B, atabrine hydrochloride (5 mgms. per kgm.). An interval of 15 minutes elapsed between A and A1, and both vagus nerves were cut 10 minutes before A1. An interval of 15 minutes elapsed between A1 and B. Electrocardiograms were taken at nos. 1 to 9.

In figure 4 may be seen the ordinary effects of such a dose of adrenaline alone in the pentobarbitalized dog, before (A) and after (A1) double vagotomy. It

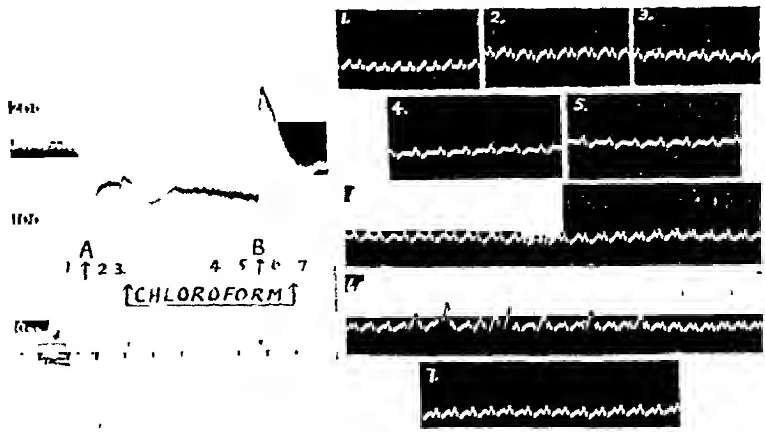

FIG 5 Dog, female, 7 2 kgm. Sodium pentobarbital anesthesia Artificial respiration Blood pressure tracing and electrocardiograms (Lead II). At A, atabrine hydrochloride (5 mgm per kgm) was injected, and at B, adrenaline (002 mgm. per kgm.). Electrocardiograms were taken at nos 1 to 7 (no. 6A is a continuation of no. 6)

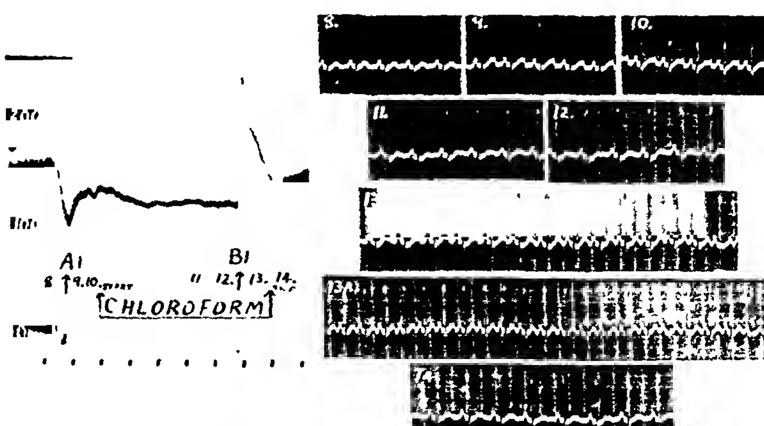

FIG. 6. Continuation of experiment shown in fig 5 An interval of 15 minutes elapsed between B (in fig. 5) and A1 Atropine sulfate (10 mgm) was injected 10 minutes before A1 At A1, atabrine hydrochloride (5 mgm per kgm) was repeated, and at B1, adrenaline (002 mgm per kgm). Electrocardiograms were taken at nos 8 to 14 (no 13A is a continuation of no 13).

should be stated that in all of these experiments electrocardiograms were taken during the entire period covering the blood pressure rise The sections shown in the figures were taken from the early segments of these records. Examinations

of records nos. 2 and 4, show that there was no significant difference in these early changes due to vagotomy. Thus, in both instances, there was an initial suppression of the P waves associated with increased T wave negativity. These effects were soon followed however, by reappearance and progressive heightening of the P waves and reversion of the T waves to a positive state. Later, in the same experiment, repetition of adrenaline (A2), after previous injection of atabrine (B) and during chloroform induced a similar pressor response, but the electrocardiograms (no. 9) showed little or no evidence of the initial alterations described above. Thus, the P wave was never suppressed, and there was no evidence of any increased T wave negativity.

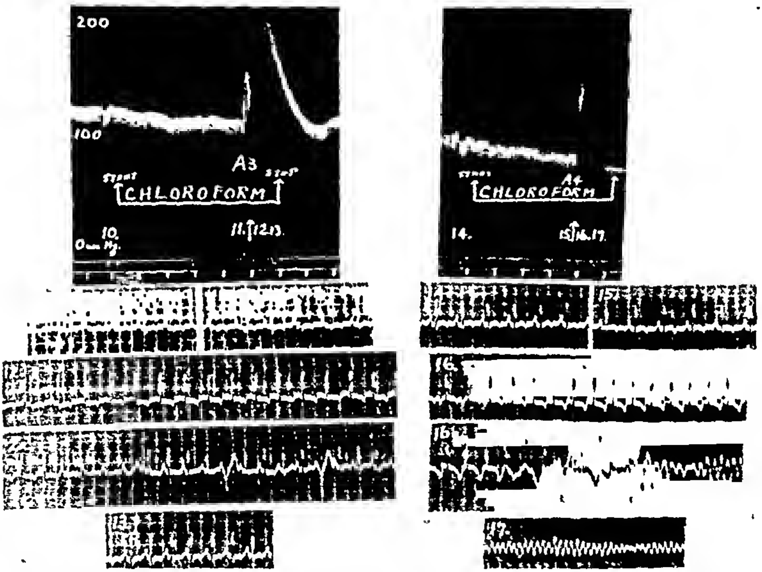

FIG. 7. Continuation of experiment shown in fig. 4. An interval of 20 minutes elapsed between A2 (in fig. 4) and A3. At A3, adrenaline (.002 mgm. per kgm.) was repeated. An interval of 20 minutes elapsed between A3 and A4, and at A4 adrenaline (.02 mgm. per kgm.) was injected. Electrocardiograms were taken at nos. 10 to 17 (nos. 12a and 16a, are respectively continuations of nos. 12 and 16).

Figures 5 and 6, confirm these latter observations, both in the animal with the vagi intact (fig. 5) and after previous injection of atropine (fig. 6). Thus, in both instances, injection of .002 mgm. per kgm. of adrenaline (B, fig. 5 and B1, fig. 6) after atabrine and during chloroform administration as shown, induces the usual pressor response, but there was complete absence of the early P and T waves changes observed with adrenaline alone. The electrocardiograms nos. 6 (fig. 5) and 13 (fig. 6) were of a uniform normal appearance. The continuations of these records (nos. 6A and 13A respectively) show in addition the appearance of several extrasystoles in the normal animal but these were not in evidence after atropinization.

Finally, figure 7, which is a continuation of the experiment shown in figure 4, shows contrastingly the effects of a similar dose of adrenaline (A3), during chloroform administration without atabrine. Thus, there is again early P wave suppression associated with increased T wave negativity (no. 12), followed by reappearance of the P wave and return of the T wave to a positive state (nos. 12A and 13). A few extrasystoles are seen (no. 12A). When a large dose of adrenaline (.02 mgm. per kgm.) was later injected under similar conditions, these characteristic early alterations in the form of the electrocardiogram are even more strikingly seen (no. 16), and were now followed by several extrasystoles and ventricular fibrillation (nos. 16A and 17). There was no evidence of any reversal of these early changes.

In addition to the above-described effects of adrenaline, the figures also show that the form of the electrocardiogram is not greatly affected by atabrine. Thus, as shown in fig. 5 (nos. 2 and 3), following injection of atabrine in the normal animal, during the fall of blood pressure, the heart rate was slightly accelerated and the T waves were more rounded than in the control (no. 1) and slightly notched. After atropinization (fig. 6 nos. 9 and 10), and again, after double vagotomy (fig. 4, nos. 6 and 7), the effects of atabrine were rather similar except that there was no evidence of cardiac acceleration.

In all of the above experiments chloroform induced slight bradycardia associated almost invariably with flattening of the T waves (fig. 5, nos. 4 and 5 and fig. 6, nos. 11 and 12). In figure 4 (nos. 7 and 8) the T wave showed a slight terminal negativity.

Discussion. The above results show clearly that the intravenous injection of atabrine can protect dogs from chloroform-adrenaline ventricular fibrillation and death. This protective action also occurs after double vagotomy or atropinization. This effect of atabrine is however exceedingly transitory even when quantities of the drug are slowly injected into the circulating blood. In this connection, it should be stated that it is known that atabrine disappears very rapidly from the blood of man after oral administration (3) and Dearborn, Kelsey, Oldham and Geiling (4) have shown that following prolonged daily oral administrations of atabrine to dogs, despite the fact that the daily excretion was only 4% of the daily dose and there were high concentrations of atabrine in other body tissues, only minute quanitities (average 0.5 mgm. per litre) could be detected in the blood at any time. This fleeting protective effect of atabrine might therefore be due to its rapid removal from the blood stream.

The data do not permit any definite conclusions concerning the mechanism of this phenomenon. Indeed, the mechanism of chloroform-adrenaline ventricular fibrillation is still obscure. It is of interest however to discuss certain possibilities.

Since it is known that atabrine induces coronary dilatation, and it has been shown in earlier studies that various other coronary dilator agents can prevent pituitary ventricular fibrillation in phenobarbitalized dogs, the question arises whether this protective action might not also be due to a similar mechanism. Indeed, Dautrebande and Charlier (5), have observed that caffeine, amyl nitrite

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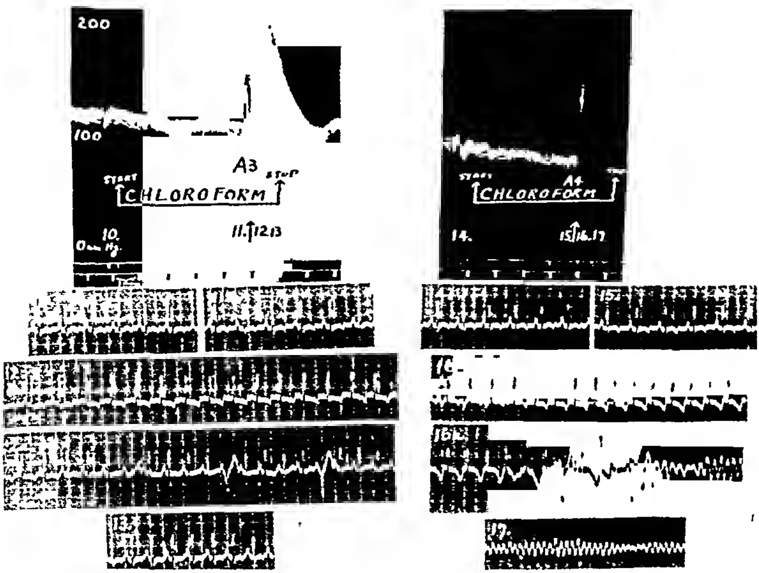

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and sodium nitrite protect the heart against benzol-adrenaline ventricular fibrillation. These authors however consider this protection to be due to the peripheral vasodilatation induced by these agents preventing the rise in blood pressure with adrenaline. One of the striking characteristics in chloroform-adrenaline ventricular fibrillation is, that it is associated with an abrupt rise in blood pressure and most investigators, including Levy (6), Bouckaert and Heymans (7), Shen (8, 9), and Shen and Marri (10), regard the rise in blood pressure following injection of adrenaline as the important factor in the production of chloroform-adrenaline ventricular fibrillation.

It is obvious from the above studies that in the presence of atabrine the blood pressure rise might be just as abrupt as in the untreated animal, but fibrillation does not occur. Hence the rise in blood pressure *per se* cannot be regarded as the initiating factor in this phenomenon.

Examination of the electrocardiograms taken during the adrenaline rise in the pentobarbitalized dog shows characteristic variations in both P and T waves concomitant with the rise in blood pressure. These changes are markedly accentuated in the presence of chloroform, but can be prevented to a large extent by administration of atabrine prior to or during chloroform. It would appear, therefore, that some action on the heart, of which these alterations are a measure, might be responsible for the onset of fibrillation. Whether or not these are due to alterations in coronary flow or to other changes in the heart cannot be definitely stated.

It is of interest to note however, that while there are abundant data in the literature to indicate that adrenaline induces mainly coronary dilatation in the dog's heart, Brodie and Cullis (11) observed several years ago, that in the isolated perfused rabbit's heart a small dose of adrenaline induces a brief initial coronary constriction followed by dilatation. More recently, in coronary perfusion experiments on the dog's heart inactivated by producing ventricular fibrillation, Katz, Lindner, Weinstein, Abramson and Jochim (12) also observed that adrenaline frequently induces a transitory coronary constriction followed by coronary dilatation. Most other investigators have concluded that adrenaline induces only coronary dilatation in these species.

Furthermore, it should be emphasized that chloroform-adrenaline fibrillation always occurs with extreme rapidity, generally within 30 seconds after the intravenous injection of adrenaline. Indeed, its development is contingent upon some early or immediate effect of adrenaline on the heart, which is not in evidence later. Thus, Shen and Marri (10) found that the *intrapericardial* injection of a large dose of adrenaline does not induce ventricular fibrillation during benzol administration, despite the fact that the blood pressure gradually rose to a high level. Moreover, in two experiments which the author recently carried out under conditions similar to those described above, it was observed that during prolonged continuous intravenous injection of adrenaline (.02 mgm. per kgm. per min.) the superimposed administration of chloroform for five minutes, failed to induce fibrillation although the blood pressure was being maintained at over 200 mm. of mercury throughout. Indeed, the fact that the electrocardiograms

taken during the adrenaline rise show marked early alterations in both P and T waves which are different from those observed later, suggest that these initial effects are due to a different mechanism.

In view of the above observations, the possibility is suggested that this protective action of atabrine might involve primarily changes in coronary blood flow. Thus, it is conceivable that the coronary dilator action of atabrine might offset either an initial reduction in coronary flow or some impairment in myocardial nutrition, induced by adrenaline under the conditions described. This however, is purely hypothetical, and the problem is being further investigated.

SUMMARY

It is shown that intravenous injections of atabrine hydrochloride can prevent the development of ventricular fibrillation following injection of adrenaline during chloroform administration in dogs anesthetized with pentobarbital. This protective action of atabrine is also demonstrable after atropinization or vagotomy.

Electrocardiograms taken during the rise in blood pressure following injection of adrenaline in the pentobarbitalized dog, show characteristic early diphasic alterations in both P and T waves. These alterations are accentuated if chloroform is being administered, but under similar conditions are antagonized by atabrine.

The possibility is suggested that this protective action of atabrine is primarily a coronary phenomenon. Thus, atabrine (a coronary dilator agent) might offset either an initial reduction in coronary blood flow or some impairment in myocardial nutrition, induced by adrenaline under the conditions described.

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BLOOD PLASMA ATABRINE LEVELS OBTAINED WITH SUPPRESSIVE AND THERAPEUTIC DOSES OF ATABRINE DIHYDROCHLORIDE

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INTRODUCTION. At the time this study was initiated, atabrine was being used as a malarial suppressive because it was the only effective drug available in large quantities, but little was known with regard to the relationships between dosage and plasma levels and between these factors and the minimal protective plasma levels. As regards the first of these relationships, studies just being completed at that time and since published (1) have demonstrated that the blood plasma levels obtained with similar doses may vary considerably from individual to individual. The present report is an extension of our knowledge concerning the relationship between dosage and plasma levels after the administration of atabrine.

PROCEDURE. The untoward reactions associated with the various doses of atabrine used in the present study have been reported previously (2). The subjects were all student medical officers unless otherwise noted. The drug was given always after the noon meal. If the drug was given twice a day, it was given after lunch and again after dinner. All blood samples were taken immediately after lunch but before the noon dose of the drug. Potassium oxalate was used as an anticoagulant. The method for the determination of atabrine was that of Brodie and Udenfriend (3). The only variation was the use of five cc. samples and duplicate determinations. The number of subjects used and the doses employed in each group were as follows: *Group A*—Nineteen men received 0.05 grams of atabrine twice a day for five days and after that 0.05 grams daily for a total of eight weeks. *Group B*—Each of this group of twenty men was given a single 0.1 gram tablet six days a week for eight weeks. *Group C*—Each of thirty subjects received 0.06 grams of atabrine dihydrochloride three times a day for five days, and then one tablet daily for a total of eight weeks. *Group D*—This group of twenty subjects received 0.06 grams daily for twenty-five days, after which the drug was discontinued and the change of plasma atabrine levels observed. *Group E*—This group of twenty-two subjects received 0.1 gram of atabrine twice a day for six days and then 0.1 gram six days a week for a total time of eight weeks. *Group F*—This group of 31 subjects received 0.1 gram twice a day for six days and then 0.1 gram daily for a total time of four weeks. *Group G*—This group of 28 men received 0.1 gram of atabrine twice a day for seven days and once a day for seven days. *Group H*—This group of 31 men received the same dose of atabrine as in Group G and, in addition, were given 5 grams of sodium bicarbonate with each dose of atabrine for the entire fourteen day period. *Group I*—These 45 subjects received the same dose of atabrine as Group G and, in addition, were given 30 cc. of 95 per cent ethyl alcohol with the noon dose of atabrine each day for the first seven days. The alcohol was diluted with an equal volume of a mixture of honey, water, oil of peppermint and oil of cloves. *Group J*—Twelve enlisted men received 0.3 grams of atabrine three times a day for five to seven days.

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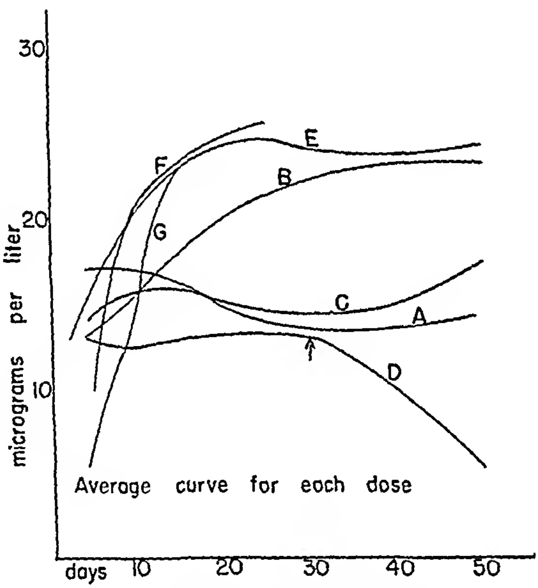
Results and discussion. The mean results for each group, except Groups H, I and J, are presented in figure 1. There was great variation in the plasma levels obtained among the various groups. Even after receiving the drug for four to six weeks the individual values ranged from less than 5 micrograms per liter to occasional values of 50 to 100 micrograms per liter. A similar variation has been reported by Shannon and collaborators (1) and by Fairley (4). This variation may be due to the peculiar pharmacology of atabrine involving as it does a marked deposition in the tissues (5, 6) and the excretion of only small amounts in the urine and feces (5, 6, 7). Since degradation in tissues probably represents the principal means of removal from the body (1), there may be many



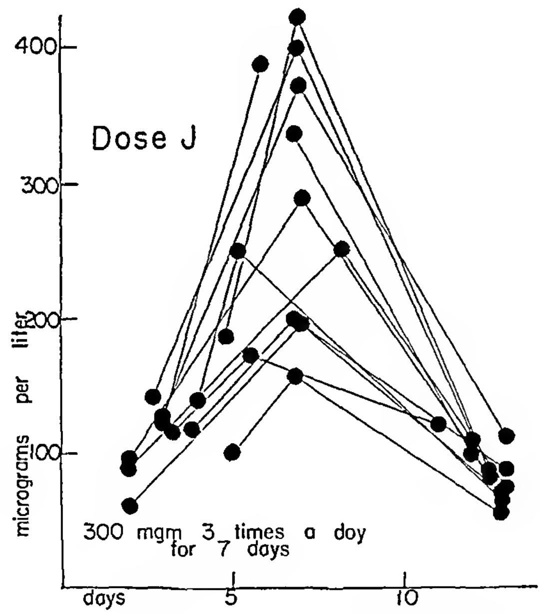
Atabrine in Plasma Fig. 1

factors involved in the plasma levels obtained and in the rate at which the plasma levels fall after the drug is discontinued. The mean plasma levels of Group B (0.1 gram six days a week) may be compared with the date of Shannon *et al.* (1). In the present study the mean level after five weeks was approximately 23 micrograms per liter as compared to a mean of 17 micrograms per liter found by Shannon (1). Part of the difference may be due to the fact that Shannon's data represent minimal values whereas in the present study blood samples were taken just before the drug was given on any day of the week and not just at Monday noon when it might be expected to be minimal. With a daily dose of 0.1 gram Fairley found (4) mean plasma levels of 23 micrograms per liter which

are the same as reported in the present study with a dose of 0.1 gram 6 days a week (Group B).

The effect of the sodium bicarbonate or ethyl alcohol on plasma atabrine levels was negligible.

It may be seen from figure 1 that the plasma levels obtained with weekly maintenance doses of 350 to 420 mg. (Groups A, C, and D) are approximately proportional to the dose administered. The relatively slow rate of excretion of atabrine is demonstrated by the mean decrease in plasma level after atabrine was discontinued (curve D in figure 1). After the atabrine was discontinued



Atabrine in Plasma Fig. 2

the average rate of fall was approximately five per cent per day and after seventeen days the level was approximately one-half the maintenance level.

The values for Groups E, F, and G represent the results of "priming" doses, that is, the administration of double doses during the first week. Compared to Group B, they show that plateau levels may be reached in approximately 14 days in this way instead of in 30 days when a constant weekly dose of the drug is used.

The data in figure 2 illustrate the high plasma atabrine levels that readily may be obtained if a large enough dose is employed. With this dose, however, the rate of decrease in plasma levels was approximately twenty per cent per day, or four times as fast as at the lower plasma levels.

SUMMARY

1. The blood plasma levels of subjects receiving the same dose of atabrine are extremely variable.

2. The group average plasma levels obtained are approximately proportional to the dose used.

3. With constant daily doses, four to five weeks are required to reach constant mean plasma levels. Similar plasma levels are reached in two weeks when the dose is doubled during the first week.

4. With low plasma levels of atabrine a reduction to one-half occurs in approximately two weeks after administration of the drug ceases. With a large dose and high plasma levels the reduction is more rapid.

5. Moderate doses of sodium bicarbonate or of ethyl alcohol do not affect the plasma levels.

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LETHAL DOSE AND AVERAGE RATE OF UPTAKE OF G-STROPHANTHIN IN THE HEART-LUNG PREPARATION OF THE DOG UNDER VARYING CONDITIONS

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It has been previously shown that in the heart-lung preparation (H.L.P.) of the dog, the lethal dose (L.D.) of g-strophanthin and of digitoxin is dependent on the rate of administration of these glycosides (1). The L.D. of g-strophanthin is determined by infusing it at a constant rate into the H.L.P. The end point of the experiment is ventricular fibrillation, the infusion being continued until ventricular fibrillation appeared. The minimal lethal dose (M.L.D.) is that lethal dose of g-strophanthin per gram heart which cannot be reduced by any further reduction in the rate of administration. *The optimal rate of administration* is the highest rate of administration at which the M.L.D. is still being determined and is a characteristic value for each glycoside. It has been previously shown that one gram of heart in the H.L.P. of the dog binds only one M.L.D. of g-strophanthin regardless of the rate of administration (1).

By dividing the M.L.D. by the experimental time, *the average rate of uptake* can thus be established. In these experiments certain experimental conditions, such as work of the heart, blood temperature, blood-volume, and heart rate were kept constant. The object of the present study was to see whether quantitative and qualitative changes of work or changes of blood volume, temperature, and heart rate have any influence on either the lethal dose, minimal lethal dose, or average rate of uptake of g-strophanthin in the heart-lung preparation of the dog.

Soluble barium salts are said to have pharmacological actions on the heart similar to those of the cardiac glycosides (2, 3). Blumenthal and Oppenheimer (4) claim that the action of barium chloride ($BaCl_2$) and of some cardiac glycosides are additive. We have tried to ascertain whether sublethal doses of $BaCl_2$ have any influence on either the L.D., M.L.D., or average rate of uptake of g-strophanthin. Kohn (5) and Weese and Wiegand (6) have shown that caffeine decreases the lethal dose of ouabain in the H.L.P. of the cat. Both these investigators used an experimental period of 20–40 minutes and thus were probably not determining the M.L.D. The cause for such a reduction in the lethal dose could either be an increase in the average rate of uptake, a decrease in the M.L.D., or a change in both these factors. We have investigated this problem and performed a number of experiments on the influence of caffeine on the L.D., M.L.D., and average rate of uptake of g-strophanthin in the heart-lung preparation of the dog.

The question has been disputed whether there are differences in the sensitivity

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of animals to the cardiac glycosides related to age. Haag and Corbell (7), who have reviewed the literature, were unable to detect significant differences in the lethal doses of tincture of digitalis, gitalin, and ouabain in cats of different age groups. Chen and Brown Robbins (8), on the other hand, have detected differences in sensitivity of etherized rabbits to ouabain related to age. We have determined the L.D. of digitoxin in anesthetized adult dogs and pups and the M.L.D. of digitoxin and g-strophanthin in the H.L.P. of pups.

Materials and Methods. G-strophanthin "Thoms" (Merck) was used. According to the manufacturers, it contains 20 per cent water of crystallization (9). All values given here have been calculated on the basis of the anhydrous glycoside. The solutions used varied between 1:15,000 to 1:500,000 g-strophanthin dissolved in 0.9 per cent saline. The digitoxin employed was a crystalline preparation;[2] 0.2 per cent stock solutions in 95 per cent ethyl alcohol were used for preparing the desired dilutions in saline.

Constant infusions were made in most experiments by means of a pump consisting of an electric motor, a gear-box and rack and pinion to push the piston of a 30 cc. B.D. syringe at a constant rate. By changing the gears, 24 different speeds could be obtained and the error in each individual determination never exceeded 5 per cent. The refilling of the syringe and readjustment of the apparatus could be done within a period of 1 to 2 minutes.

The determination of the lethal dose of digitoxin in intact dogs and pups was made on animals anesthetized with 30 to 40 mg. of sodium pentobarbital per kg. body weight given intraperitoneally. The pups were animals still possessing their milk teeth, and no special effort was made to determine their exact age. After anesthesia had set in, the trachea, left common carotid and left femoral vein were isolated. Artificial respiration was given by means of a Palmer respiration pump. Arterial blood pressure was recorded from the left common carotid artery by means of a mercury manometer. Continuous infusion of 1:50,000 crystalline digitoxin in saline was made into the left femoral vein by means of a Marriotte tube. In all, 6 pups and 6 adult dogs were used for this series of experiments.

In the H.L.P. of pups the M.L.D. of both digitoxin and g-strophanthin was determined by the method used for adult dogs. Out of 6 experiments only 2 were satisfactory with digitoxin, while with g-strophanthin 4 out of 7 experiments were successfully concluded.

In these experiments the hearts of the pups were perfused with blood from adult dogs, otherwise the experimental conditions were similar to those previously described (1). (For further details on experimental conditions see table 2.)

Results. 1. *Work of the heart.* Work of the left heart was determined from the systemic output (output of the left heart minus coronary flow) and the arterial pressure. At the beginning of each experiment the systemic output and resistance were adjusted to give the desired work performance. Four to eight checks were made during the course of each experiment and whenever necessary, the height of the inflow vessel was readjusted so as to give the desired work performance.

In the series of experiments published previously, the work performance of the left heart was 0.65–0.72 kilogram-meters per minute, the peripheral resistance was 75 mm. Hg and the systemic output about 400 cc. per minute. The experimental data are represented by a heavy line in figures 1, 2, and 3.

In order to see whether a shift from pressure work to volume work would modify the L.D., M.L.D., and average rate of uptake of g-strophanthin, the peripheral resistance was reduced from 75 to 40 mm. Hg and the systemic output

[2] Kindly supplied by Hoffmann-LaRoche, Basel, Switzerland.

increased from 400 to about 550 cc., representing a work performance of the left heart of about 0.7 kilogram-meters per minute. From figure 1A it can be seen that a change from pressure to volume work has not changed significantly either the L.D. or M.L.D. of g-strophanthin (6 experiments). Furthermore, from figure 1B, it is also clear that the average rate of uptake is about the same regardless of the type of work performed.

In ten experiments, the influence of the reduction of the work of the left heart

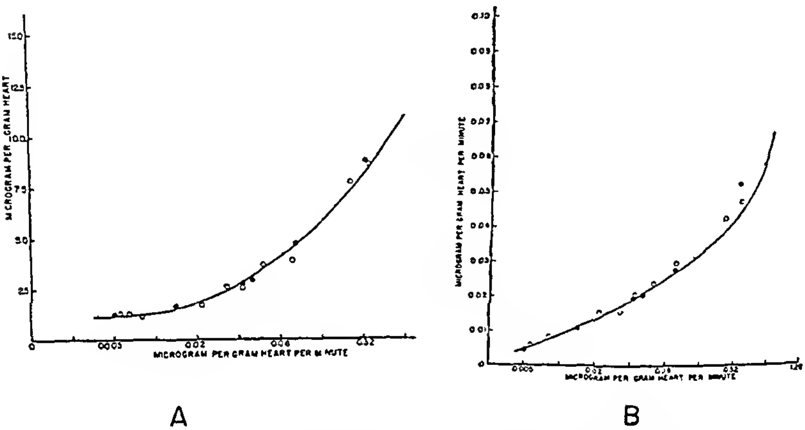


FIG. 1A. THE INFLUENCE OF CARDIAC WORK ON THE LETHAL DOSE OF G-STROPHANTHIN IN THE H.L.P.

Abscissa = rate of administration, ordinate = lethal dose

FIG. 1B. THE INFLUENCE OF CARDIAC WORK ON THE AVERAGE RATE OF UPTAKE OF G-STROPHANTHIN IN THE H.L.P.

Abscissa = rate of administration, ordinate = average rate of uptake

Solid curve: The lethal dose and average rate of uptake of g-strophanthin when the heart is performing resistance work. Data published previously (1) (26 experiments). The experimental conditions were: work of the left ventricle, 0.65–0.72 kilogram-meters per minute; peripheral resistance, 75 mm. Hg; systemic output, 380–420 cc. per minute; blood temperature, 38.8–39.2°C; blood volume, 850–900 cc.

Full circles: The lethal dose and average rate of uptake of g-strophanthin when the heart is performing volume work. Work of the left heart 0.68–0.72 kilogram-meter; peripheral resistance = 40 mm. Hg systemic output, 500–550 cc. per minute; blood temperature, 38.8–39.2°C; blood volume 850–900 cc.

Open circles: The lethal dose and average rate of uptake of g-strophanthin when the work performance of the heart is low. Work of the left heart = 0.32–0.35 kilogram-meters; peripheral resistance, 30 mm. Hg; systemic output, about 300 cc.; blood temperature, 38.8–39.2°C; blood volume, 850–900 cc.

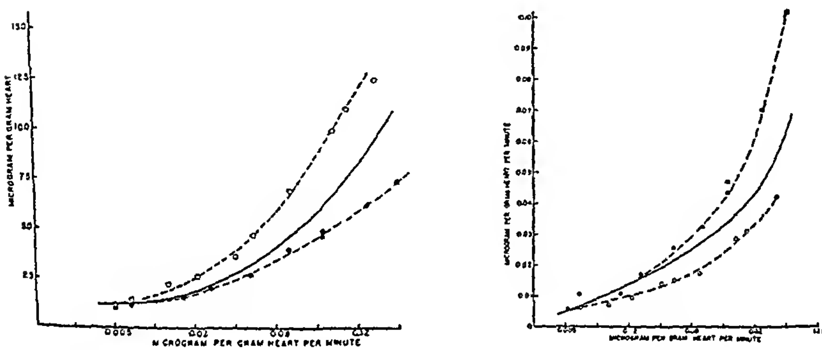
on the L.D., M.L.D., and average rate of uptake of g-strophanthin was studied. The peripheral resistance was decreased to 30 mm. Hg and the systemic output to about 300 cc. per minute, so that the work of the left heart was about 0.35 kilogram-meters per minute. The mean aortic pressure was about 70 mm. Hg and was thus adequate for an appropriate coronary flow.

From figure 1A it can be seen that the reduction in the work of the left heart did not significantly influence either the L.D. or M.L.D. of g-strophanthin;

while figure 1B shows that the average rate of uptake is also the same whether the work of the heart is high or low.

2. *Blood volume.* In the series of experiments published previously (1), the blood volume was 850–900 cc. Ten experiments were performed with a blood volume of 480–520 cc.

The reduction in blood volume leads to a decrease of the L.D. of g-strophanthin when high rates of administration are used. But, as the rate of administration is diminished, the L.D. approaches that determined with the higher blood volume. At a rate of 0.015 microgram per gram heart per minute, the lethal dose is the



A B

FIG. 2A. THE INFLUENCE OF BLOOD VOLUME AND BLOOD TEMPERATURE ON THE LETHAL DOSE OF G-STROPHANTHIN IN THE H.L P.

Abscissa = rate of administration; ordinate = lethal dose

FIG. 2B. THE INFLUENCE OF BLOOD VOLUME AND BLOOD TEMPERATURE ON THE AVERAGE RATE OF UPTAKE OF G-STROPHANTHIN IN THE H I .P.

Abscissa = rate of administration; ordinate = average rate of uptake

(a) Solid curve: Control data, representing the same data as in figures 1A and 1B.

(b) ● ----- ●: The effect of reducing the blood volume on the lethal dose and average rate of uptake of g-strophanthin. Blood volume, 480–520 cc., otherwise, the experimental conditions were the same as those in figures 1A and 1B

(c) O ----- O: The effect of reducing the blood temperature on the lethal dose and average rate of uptake of g-strophanthin Blood temperature, 35 0–35 3°C; otherwise, the experimental conditions were the same as those in figures 1A and 1B.

same regardless of the blood volume (see fig. 2A). A reduction in the blood volume, therefore, does not affect the M.L.D. of g-strophanthin, although it changes the L.D.

A reduction of the blood volume increases *the average rate of uptake* when rates of administration greater than the *optimal* are being employed. However, when the rates become equal to or less than the optimal rate of administration, the average rate of uptake is the same whether a high or low blood volume is used (fig. 2B).

3. *Temperature of the blood.* In the experiments published previously (1) the

blood temperature was kept at 38.8–39.2° C. We have performed 10 experiments with a blood temperature of 35.0–35.3°C. In figure 2A the lethal doses of g-strophanthin determined at 35.0–35.3°C. are compared with those determined at 38.8–39.2°C. It is clear from this figure that at the same rate of administration the lethal dose is higher when the blood temperature is low.

Furthermore, a reduction in the blood temperature has shifted the optimal rate of administration from a value of 0.012 to one of about 0.006 micrograms per gram heart per minute.

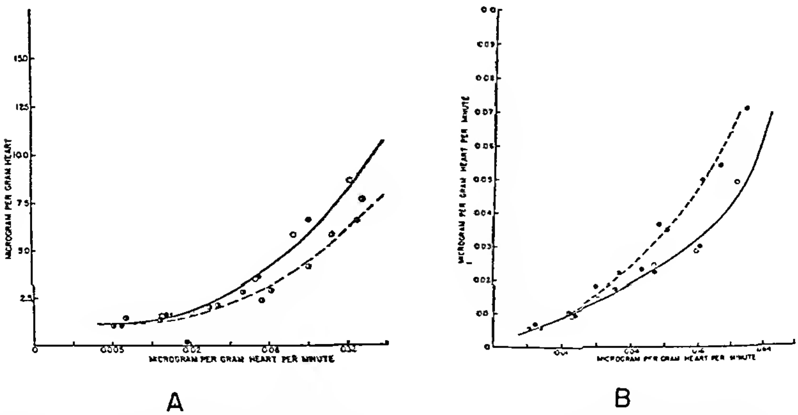


FIG. 3A. THE INFLUENCE OF EPINEPHRINE HYDROCHLORIDE, BARIUM CHLORIDE, AND CAFFEINE AND SODIUM BENZOATE ON THE LETHAL DOSE OF G-STROPHANTHIN IN THE H.L.P.

FIG. 3B. THE INFLUENCE OF EPINEPHRINE HYDROCHLORIDE, BARIUM CHLORIDE, AND CAFFEINE AND SODIUM BENZOATE ON THE AVERAGE RATE OF UPTAKE OF G-STROPHANTHIN IN THE H.L.P.

Solid curve: Control data, representing the same data as in figures 1A and 1B.
Open circles: The effect of repeated doses of epinephrine hydrochloride (20 micrograms every 15 minutes) on the lethal dose and average rate of uptake of g-strophanthin in the H.L.P.
Full circles: The effect of sublethal doses of barium chloride on the lethal dose and average rate of uptake of g-strophanthin in the H.L.P.
◑----◑: The effect of caffeine and sodium benzoate (0.5 gram) on the lethal dose and average rate of uptake of g-strophanthin in the H.L.P. Experimental conditions the same as those represented by the solid curve.

The M.L.D. does not seem to be appreciably changed by a reduction in the blood temperature (fig. 2A).

From figure 2B it can be seen that a lowering of the blood temperature from 39 to 35°C. has resulted in a decrease in the average rate of uptake of g-strophanthin in the H.L.P. of the dog.

4. *Epinephrine and heart rate.* These experiments were designed to see whether the changes in heart rate and other metabolic and hemodynamic changes produced by epinephrine have any influence on the L.D. and average rate of uptake of g-strophanthin. At a blood temperature of 39°C. the heart-rate of H.L.P. of the dog varied between 138 and 192, or an average of 178±12 beats per minute. All heart rates were counted about 15 minutes after the com-

pletion of the preparation. In 5 experiments, while the lethal dose of g-strophanthin was being determined by the continuous infusion method, 20 micrograms of epinephrine hydrochloride were injected every 15 minutes into the venous reservoir. This resulted in a maximum increase in heart rate of about 40–60 beats per minute and lasted for about 8 – 20 minutes.

From figure 3A it can be seen that the doses of epinephrine employed did not significantly change the L.D. Furthermore, the data presented here as well as previous observations (1) show that the M.L.D. of g-strophanthin is not changed significantly by epinephrine.

5. *Barium chloride and spontaneity.* Barium chloride in sufficient doses produces ventricular fibrillation. We were interested to see whether the effects of sublethal doses of $BaCl_2$ have any influence on the lethal dose of g-strophanthin in the H.L.P. of the dog. In 3 heart-lung prepations the lethal dose of $BaCl_2$ was found to be 0.75, 0.79, and 0.90 mg., an average of 0.81 mg. of $BaCl_2$ per gram of heart.

In 5 experiments the influence of sublethal doses of $BaCl_2$ (0.24 – 0.67 mg. $BaCl_2$ per gram of heart) on the lethal dose of g-strophanthin was determined. The total amount of $BaCl_2$ to be added was introduced into the venous reservoir and 10 – 15 minutes later the infusion of g-strophanthin was started. From figure 3A it can be seen that in 4 out of the 5 experiments neither the L.D. or M.L.D. of g-strophanthin was appreciably affected by the presence of $BaCl_2$. However, one experiment showed a L.D. of 0.133 micrograms of g-strophanthin. The dose of $BaCl_2$ employed in this experiment was 0.67 mg. per gram heart, a dose which is very close to the lethal dose of $BaCl_2$ itself. It is thus possible that in this experiment the $BaCl_2$ and not g-strophanthin was the cause of the ventricular fibrillation. The average rate of uptake is apparently not influenced by sublethal doses of $BaCl_2$ (see fig. 3B).

6. *Caffeine.* Caffeine and sodium benzoate in a dose of 0.5 gram was added into the venous reservoir. This dose resulted in a concentration of about 1:1800 of caffeine and sodium benzoate and was already within the toxic range of this drug since this concentration frequently resulted in a rise in right auricular pressure and a reduction in the systemic output. About 10 minutes after the addition of caffeine sodium benzoate the constant infusion of g-strophanthin was started. From figure 3A it can be seen that the L.D. of g-strophanthin was reduced only when rates of administration greater than the optimal were being employed, while the M.L.D. of g-strophanthin was not significantly changed. The cause of this reduction in the L.D. is probably due to an increase in the average rate of uptake (fig. 3B).

7. *Age.* Differences in sensitivity to cardiac glycosides due to age would be very important for most types of digitalis assay, since animals of an unknown age are usually employed in such determinations.

We have performed with digitoxin 6 experiments each in anesthetized adult and young dogs. It was clear from the start that the control of the rate of administration was a most important factor, since a disregard of this point would give the most variable results. From table 1 it can be seen that in the dog, age

of the animal does not play an important rôle in determining sensitivity to digitoxin. Out of 6 such experiments in which the M.L.D. of digitoxin in the H.L.P.

TABLE 1

The lethal dose of crystalline digitoxin in young and adult intact anesthetized dogs

NUMBER	WEIGHT	RATE OF ADMINISTRATION	LETHAL DOSE OF DIGITOXIN	EXPERIMENTAL TIME
		Adult dogs		
	kg.	*mg. per kg. per hour*	*mg. per kg.*	*min.*
1	7.80	0.198	0.526	160
2	9.22	0.158	0.303	115
3	7.05	0.219	0.446	122
4	6.25	0.243	0.389	96
5	4.75	0.222	0.581	157
6	4.70	0.213	0.515	145
Average	6.63	0.209 ±0.0118	0.460 ±0.0416	132.5 ±10.4
		Young dogs		
1	2.65	0.192	0.355	111
2	2.47	0.198	0.364	110
3	5.70	0.168	0.735	260
4	2.86	0.160	0.518	186
5	4.05	0.216	0.604	175
6	2.78	0.186	0.313	101
Average	3.42	0.187 ±0.0084	0.482 ±0.0675	157 ±25.8

TABLE 2

The determination of the minimal lethal dose of digitoxin and of g-strophanthin in the H.L.P. of puppies

Blood volume = 850–900 cc. Output of left heart = 400–420 cc. per minute. Blood temperature = 38.8–39.1°C. Resistance = 75 mm. Hg. Work of left heart = 0.68 – 0.720 kilogram-meters per minute.

NUMBER	BODY WEIGHT	HEART WEIGHT		RATE OF ADMINISTRATION	LETHAL DOSE	EXPERIMENTAL TIME	REMARKS
		Uncorrected	Corrected				
	kg.	*g.*	*g.*	*gamma per gram heart per minute*	*gamma per gram heart*	*min.*	
1	3.90	41	32.7	0.0246	5.23	213	Digitoxin
2	3.65	38	30.6	0.0210	5.78	275	Digitoxin
1	2.31	22		0.00817	1.135	139	G-Strophanthin
2	3.24	44	27.1	0.00791	1.298	164	G-Strophanthin
3	3.50	39		0.00708	1.197	169	G-Strophanthin
4	3.15	37.2	26.5	0.00591	1.096	185	G-Strophanthin

of pups was determined, only 2 could be considered satisfactory, since the long experimental periods necessary for such a determination resulted in a premature

lung edema. However, as can be seen from table 2, both the values determined agree fairly well with the values determined on adult dogs and published previously (1). In seven experiments the M.L.D. of g-strophanthin was determined on the H.L.P. of pups, 4 were satisfactory, and here again the values of the M.L.D. of g-strophanthin do not differ significantly from those determined on the H.L.P. of adult dogs.

DISCUSSION. Under the variable experimental conditions which have been studied here, the M.L.D. of g-strophanthin in the H.L.P. of the dog is a fairly constant value. The differences have been in the lethal dose determined with higher rates than the *optimal rate of administration*. Since under these varied experimental conditions the M.L.D. is constant, the observed differences in the lethal dose are probably due to changes in the *average rate of uptake* of g-strophanthin.

From our data the averge rate of uptake is not influenced by either qualitative or quantitative changes in heart-work, increase in heart rate, and other hemodynamic and metabolic changes produced by epinephrine.

A change in blood volume from about 900 cc to 500 cc resulted in an increase of the average rate of uptake only with rates of administration greater than the optimal. At rates less than the optimal, M.L.D. and average rate of uptake were the same regardless of the blood volume (fig. 2). It is conceivable that at the same rate of administration, the concentration of the glycoside might rise more rapidly and be actually higher when the blood volume is low. It is probable therefore that at rates higher than the optimal the concentration does influence the average rate of uptake. However, at or below the optimal rate of administration the concentration of the glycoside in the blood obviously does not influence either the M.L.D. or the average rate of uptake of g-strophanthin. In other words, in the heart-lung preparation of the dog, the determining factor for the M.L.D. is not the concentration but the absolute amount of the glycoside in the circulating blood.

The lowering of the blood temperature resulted in a decrease in the average rate of uptake of g-strophanthin. This decrease might be due to the reduction in the temperature or the decrease in heart rate. As the increase in heart-rate produced by epinephrine did not influence the average rate of uptake of g-strophanthin, it is at least probable that the decrease in heart-rate, produced by lowering the blood temperature, is not the decisive factor in the reduction of the average rate of uptake of this glycoside.

The experimental results on the influence of temperature changes on the speed of reaction of digitalis glycosides have been utilized to determine whether the reactions of these glycosides with the heart muscle are of a chemical or physical nature (10). Vant Hoff's law states than an increase of 10°C. should increase the speed of reaction by 200–300 per cent (11). The experimental results of Gunn (12) on the Langendorf heart of the rabbit do not show such a relationship between temperature change and the time for systolic arrest of the heart. Our experiments indicate that with the highest rates of administration a temperature

increase of about 4°C. resulted in an increase in the average rate of uptake of about 25 per cent. However, the influence of temperature on the optimal rate of administration is more marked. Raising the blood temperature by about 4°C. led to an increase of this value from 0.006 to about 0.012 micrograms of g-strophanthin. From these figures it follows that an increase of about 250 per cent in the optimal rate of administration would probably occur if the temperature were raised by 10°C.

The uptake of cardiac glycoside by the heart is a complicated process involving a number of steps regarding which very little is known. Any influence of temperature changes on the average rate of uptake is the resultant of changes which may occur in one or more of these reactions. To decide from temperature data whether a reaction is of a chemical or physical nature is not feasible, especially so with the reaction between cardiac muscle and glycoside. The nature of this reaction is so obscure at present that no final conclusions can be drawn.

The effects of sublethal doses of barium chloride and g-strophanthin are not additive in the H.L.P. of the dog. Wégria et al. (13) have shown that the fibrillation threshold of direct current is not influenced by toxic doses of digitalis glycosides. From this and our experiments with barium chloride, it can be concluded that the mechanism of action in the production of ventricular fibrillation is not always the same.

The influence of large doses of caffeine has been a decrease in the lethal dose when rates greater than optimal are employed. This confirms the data of both Kohn (5) and Weese and Wiegand (6). However, it is clear from our data that this is only true when rates of administration higher than the optimal are being employed.The M.L.D. of g-strophanthin is not changed by caffeine and it is quite probable that the increase in the average rate of uptake is the determining factor in the observed reduction of the lethal dose of g-strophanthin. This opinion was expressed by Kohn (5) but was not supported by experimental evidence. No direct experiments have been performed to test the influence of changes in coronary flow on the L.D. and average rate of uptake of g-strophanthin in the isolated heart of the dog. However, the data obtained with epinephrine, and changes in the work of the isolated heart, procedures which markedly change coronary flow, do not influence either the L.D., M.L.D., or average rate of uptake of g-strophanthin by the H.L.P. It is thus probable that coronary flow changes do not greatly modify these values.

Our experiments on intact anesthetized dogs indicate that age differences in sensitivity to digitoxin cannot be demonstrated. Furthermore, the M.L.D. of both digitoxin and g-strophanthin as determined in the H.L.P. of pups is not significantly different from that of adult dogs. Our data thus confirm and extend those of Haag and Corbell (7). On the other hand, Chen and Brown Robbins (8) have demonstrated age differences in sensitivity to g-strophanthin in rabbits. This data, however, did not take into account the possible influence of the rate of administration of the glycoside. In a second series of experiments Brown Robbins and Chen (14) have demonstrated significant age differences in sensitivity to g-strophanthin in both dogs and rabbits when the rate of administration

was carefully controlled. However, the rate of administration employed by Brown Robbins and Chen (14) was about 3 and 5 micrograms of g-strophanthin per kg. per minute for rabbits and dogs respectively.. This would produce death of the animal in approximately 25 to 50 minutes in rabbits and 25 to 35 minutes in dogs. This experimental time is probably too short to determine the M.L.D. of g-strophanthin. A final settlement of this problem can only be accomplished by comparing the M.L.D. of g-strophanthin in young and adult animals.

The outstanding finding of the previous publication (1) and of the present series of experiments is the constancy of the minimal lethal dose of g-strophanthin in the H.L.P. under the most variable conditions. It follows from this that for any comparative study of the activity of cardiac glycosides or the modification of their toxicity by physiological, pharmacological, or pathological changes, the M.L.D. rather than the L.D. should be used as a basis of comparison. The factors affecting the average rate of uptake and the L.D. are the rate of administration of the glycoside, blood temperature, and blood volume, and for any comparative experiments in the H.L.P. these factors must be carefully controlled

SUMMARY

Qualitative and quantitative changes in heart work of the H.L.P. do not significantly influence either the lethal dose, minimal lethal dose, or average rate of uptake of g-strophanthin in the H.L.P. of the dog. A decrease in blood volume decreases the lethal dose and increases the average rate of uptake when rates higher than the *optimal* rate of administration of g-strophanthin are employed. The minimal lethal dose is not changed by a change in blood-volume.

A reduction of the blood temperature increases the lethal dose and decreases the *average rate of uptake* and *optimal rate of administration.* The minimal lethal dose of g-strophanthin is not influenced by a change in the blood temperature from 35 to 39°C.

Sublethal doses of barium chloride, as well as the increase in heart rate and other metabolic changes produced by epinephrine do not change the L.D., M.L.D., or average rate of uptake of g-strophanthin in the heart-lung preparation of the dog.

Caffeine and sodium benzoate increases the average rate of uptake but does not influence the M.L.D. of g-strophanthin.

No differences in sensitivity to digitoxin can be detected between intact anesthetized (sodium pentobarbital) pups and adult dogs. The minimal lethal dose of g-strophanthin and digitoxin determined in the H.L.P. of young dogs does not appear to be significantly different from the M.L.D. determined on the H.L P. of adult dogs.

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THE RELATIONSHIP BETWEEN THE LIPOID AFFINITY AND THE INSECTICIDAL ACTION OF 1,1-bis (p-FLUOROPHENYL) 2,2,2-TRICHLOROETHANE AND RELATED SUBSTANCES[1]

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Two hypotheses concerning the mechanism of the insecticidal action of 1,1-bis(p-chlorophenyl) 2,2,2-trichloroethane (DDT) (I) have been published. Läuger et al. (1) suggest that the toxic component of the molecule is the linked p-chlorophenyl rings which block an enzyme system necessary for the proper functioning of the insects' nervous system. The trichloromethyl group according to Läuger causes the molecule to have a great affinity for lipoids, thus allowing the toxic component to be concentrated at its site of action, namely the nerve lipoids.

The mechanism proposed by Martin and Wain (2) assumes that the chlorophenyl rings confer lipoid solubility while the remainder of the molecule is responsible for the toxicity by liberating hydrochloric at the "vital centers". The evidence for the Martin-Wain hypothesis rests upon the relative ease with which the in vitro dehydrochlorination of DDT and similar compounds is accomplished by alcoholic alkali. There are many objections to the Martin-Wain hypothesis. Läuger (1) and Mylius and Koechlin (6) reported two compounds (II and III) which approach DDT in insecticidal potency although neithercompound is capable of dehydrochlorination. They also reported that dehydrochlorinated DDT (IV) had insecticidal action although it was weaker and more selective in action than DDT. Busvine (3) published data on eight compounds related in structure to DDT. He compared their toxicities to the ease of dehydrochlorination and lipoid solubility. Busvine concluded that his data supported neither hypothesis since he found no correlation between toxicity, ease of dehydrochlorination and lipoid solubility. It should be noted, however, that the variation between the order of increasing ease of dehydrochlorination and incrasing toxicity was greater than that between the order of increasing fat solubility and increasing toxicity. Further Busvine determined the total solubility of the molecule which may not be the determining factor in its "in vivo" lipoid affinity. As Busvine suggests the shape and size of the molecule may be important.

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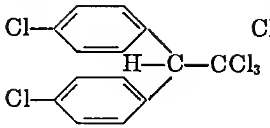

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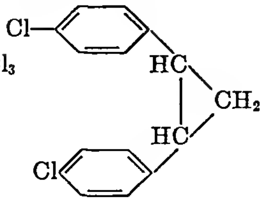

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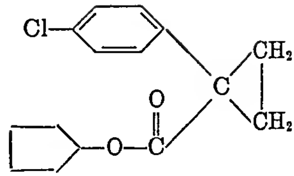

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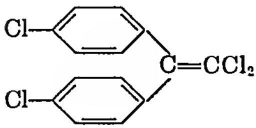

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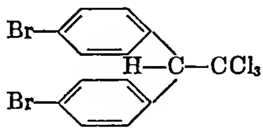

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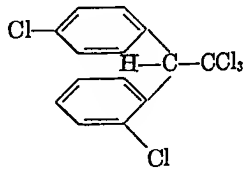

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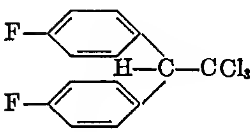

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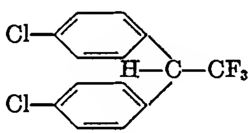

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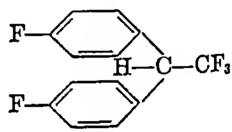

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Läuger's suggestion is an extension of the Meyer-Overton theory of the mechanism of transportation and "storage" of the general anesthetics. He presented evidence to show that DDT acts on the insects nervous system (1). Yeager and Munson (4) and Roeder and Weiant (5) have confirmed this observation. Since several para-bridged 4,4′-dichlorodiphenyl structures were shown to be very toxic when ingested by insects (1), Läuger postulated that this structure acted by blocking some enzyme system in the insect physiology. The chloroform residue he claimed acted as a lipophilous group which caused the molecule to have an affinity for lipoids just as chloroform does. This resulted in the compound being accumulated at its site of action in the nerve lipoids. To test this hypothesis twenty-four compounds were synthesized in which the trichloromethyl group of DDT was replaced by radicals derived from compounds known to function as general anesthetics (1, 6). Radicals derived from thirteen anesthetics were used and in all cases the compounds showed activity as contact insecticides. Recently evidence has been accumulating in the literature which indicates that the activity of these insecticides is correlated with their lipoid affinity. Wigglesworth (7) and Case (8) reported cases of mild DDT intoxication in humans. The symptoms were largely psychic in nature and persisted for at least 30 days after the administration of DDT was terminated. This is presumptive evidence for lipoid storage and subsequent release of the compound. Further, Woodward et al. (9) in a pharmacological study, reported that DDT and 1,1-bis(p-bromphenyl) 2,2,2-trichloroethane (V) were stored in the intraperitoneal and subcutaneous fat of dogs fed these compounds. Levels as high as 5 mgm./gm. of

fat were reached in some of these dogs. The level found in the fat was much higher when DDT was fed in corn oil than when it was fed in solid form. This indicates that DDT passed through the intestinal wall more easily when intimately associated with fat. DDT metabolites could be demonstrated in dog urine 24 days after the administration of DDT was stopped. This indicates continual release of DDT from the body stores, which are, presumably, in the fat depots. Further DDT was found to pass into the milk of a bitch to which it was fed and 24 hours after the feeding of a single 50 mgm./kilo dose of o-p DDT (VI) it could be detected in the milk. Telford and Guthrie (10) reported that the milk from either goats or rats fed DDT was toxic to rats to which it was fed. The toxic symptoms were typical of DDT poisoning and it was found that the toxic material was concentrated in the fat of the milk.

Kirkwood and Dacey (11) synthesized the compounds 1,1-bis(p-fluorophenyl) 2,2,2-trichloroethane (VII), 1,1-bis(p-chlorophenyl) 2,2,2-trifluoroethane (VIII) -and 1,1-bis(p-flurophenyl) 2,2,2-trifluoroethane (IX). Insecticidal tests showed that VII was approximately one-half as active as DDT while VIII and IX were practically inactive (11, 15). The trace of activity in VIII and IX was no doubt due to the presence of traces of the parent compounds (DDT and compound VII respectively) which have high activity. These data tend to support Läuger's hypothesis in view of Henne's (12) observation that fluoroform has no physiological action. Guinea pigs can be kept for 1 hour in an atmosphere of 80 per cent fluoroform and 20 per cent oxygen without showing any effect.

The purpose of this report is to present evidence to show that 1,1-bis(p-fluorophenyl) 2,2,2-trichoroethane (VII) upon ingestion accumulates in the body lipoids and that 1,1-bis(p-chlorophenyl) 2,2,2-fluoroethane (VIII) fed at the same level could not be detected in the perirenal fat of rats receiving it. These results would be expected under Läuger's postulation.

EXPERIMENTAL. *Materials and methods.* Compounds VII and VIII were prepared by the procedures of Kirkwood and Dacey (11).

Four groups of adult female rats were placed on experiment. Each group was housed in a separate cage and each group received stock ration and water ad libitum. The first (control) group consisted of two animals and received no supplement. The second group consisted of two animals which were given sodium fluoride, in aqueous solution, at the rate of 5 mgm./kilo/day. The third and fourth groups of four animals each received 50 mgm./kilo/day of compounds VIII and VII respectively. This was administered orally by dropper in corn oil solution (eight drops/day) One animal in the fourth group developed a tumor early in the experiment and was destroyed. With the exception of the animals in group 2 which developed mottled incisors due to the high intake of fluoride ion, none of the animals showed any symptoms of a toxic nature. All animals were maintained on the supplements for 75 days at the end of which time they were sacrificed. The perirenal fat was carefully removed and collected by lots for a pooled sample for fluorine analysis. The pooled samples were dried in a vacuum dessicator and extracted with diethyl ether for a period of 24 hours in a Soxhlet extractor. Each ether extract was washed twice with an equal volume of redistilled water to remove any traces of inorganic fluorine present. The ether solutions were then placed in a 110° oven to remove the ether and moisture from the fat.

Method of analysis. Since DDT is absorbed and stored in the body fat in unaltered form (9), it was assumed that compounds VII and VIII, if stored, would also be unaltered. Thus they could he reliably estimated by determining the ether extractable fluorinc in the fat. No ether extractable fluorine was found in the fat of control rats or in the fat of the rats in group 2 which were fed the inorganic fluoride. Kirkwood and Dacey (11) demonstrated that the fluorine present in compounds VII and VIII was quantitatively liberated as inorganic fluoride when the compounds were fused in a peroxide bomb. It was found that the Armstrong (13) modification of the Willard and Winter (14) method adapted to the photoelectric colorimeter could be used directly on the neutralized filtrates from the peroxide fusion. High concentrations of chloride ion have been found to interfere with the titration method of determining fluoride (13). This did not interfere in the colorimetric method however. A lower value for the colorimeter constant was obtained when high levels of chloride ion were present hut when log difference values were plotted against fluoride con-

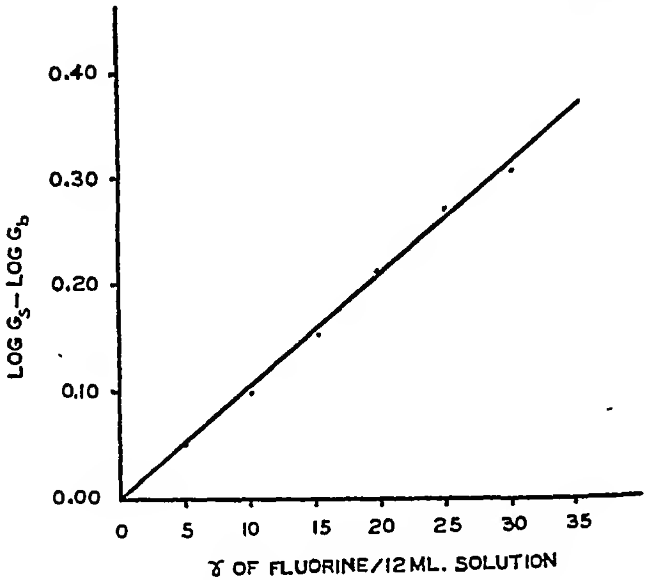

FIG. 1. CONCENTRATION OF FLUORIDE IN 8 PER CENT NaCl SOLUTION PLOTTED AGAINST LOG DIFFERENCE VALUES

centration a straight line resulted. The slope of the linc did not vary when concentrations of from 5 to 20 per cent sodium chloride were present. Figure 1 shows the range of proportionality for the Evelyn colorimeter with fluoride standards made up in 8 per cent salt solution. The range is from 0–30γ in 12 ml. solution. At higher fluoride concentrations the points do not lie on a straight line. The use of neutralized solutions from the peroxide fusion resulted in an alteration in the color tinge of the Thorium-Alizarin lake. However, the ahsorption maximum was not altcred and the hleaching of the lake by fluoride ion was not interfered with. If the solution ohtained from the fusion of 0.4 gm. of corn oil was used as a hlank excellent rccoveries of fluorine were obtained from corn oil solutions of compound VII (tahle 1). Further it was found that as little as 0.05 mg. of compound VIII per sample could be detected hy this procedure.

Apparatus. The colorimetcr used for all determinations was an Evelyn equipped with a no. 520 filter. The peroxide homb used was a Parr model AC-1.

Fusion procedure. Five gm. of sodium peroxide (reagent) and 0.5 gm. of potassium perchlorate were weighed into the bomb cup, 0.4 gm. of fat were added and the whole mixed thoroughly with a glass rod. The Bomb was then assembled, ignited and cooled. The contents of the bomb were dissolved in 50 ml. of water,[2] the solution was brought to a boil and filtered. The pH was adjusted between 2.5–3.5 (glass electrode) by the addition of concentrated HCl (about 11 ml.). The solution was then made up to 100 ml. and fluorine was determined on 10 ml. aliquots of this solution.

Reagents. Alizarin Red S (National medicinal); Thorium nitrate $12H_2O$; Redistilled monochloracetic acid; Sodium hydroxide (reagent).

Color reagent. 6.2 mgm. of Alizarin Red S and 9.4 mgm. of Thorium Nitrate $12H_2O$ were dissolved in 25 ml. of distilled water. To this was added 25 ml. of a 1 M solution of monochloracetic acid half neutralized with reagent NaOH. This reagent should not be used if it is over 24 hours old.

Analysis procedure. A blank prepared from the fusion of 0.4 gm. corn oil was run with each determination. Ten ml. aliquots of the solutions to be analyzed are placed in Evelyn tubes. Two 10 ml. aliquots of the blank were included in each run. To one of these aliquots 200γ of fluoride ion (as sodium fluoride) were added. Two ml. of the color eagent were added to each tube, the tubes were shaken and allowed to stand for 15 minutes at the end of which time they were read on the Evelyn colorimeter. The blank containing

TABLE 1

Recovery of fluorine from corn oil solutions of compound VII (0.4 gm. samples of oil used in each case)

FLUORINE ADDED AS COMPOUND VII	FLUORINE FOUND	ERROR IN FLUORINE FOUND	RECOVERY
γ	γ	γ	%
22.0	22	0.0	100
18.0	17	−1.0	94
13.0	12	−1.0	92

200γ fluoride ion was set at 100 (520 filter) and the readings of the other tubes were noted.

Calculation. The concentration of fluoride ion in the sample solutions is given by the expression $C = K (\log G_s - \log G_b)$. Where G_s is the galvanometer reading of the sample and G_b is the galvanometer reading of the blank, K is the calibration constant determined by the analysis of known solutions of compounds VII and VIII in corn oil.

RESULTS. The results are shown in table 2. No fluorine could be detected in the fat of rats fed compound VIII (group III) which contains the trifluoromethyl group. Even when three 0.4 gm. samples of fat from group III were fused, combined, neutralized and evaporated to 100 ml. no fluorine could be detected. In order to show that small quantities of fluorine could be recovered three 0.4 gm. samples of fat, containing a total of 18γ of fluorine as compound VIII, were fused. An 80 per cent recovery of the added fluorine was obtained. In contrast, compound VII which contained the trichloromethyl group was found to accumulate to the extent of 3.2 mgm/gm. of fat when fed to rats.

DISCUSSION. It is interesting that the substitution of a trifluoromethyl group for a trichloromethyl group in DDT should cause a loss of insecticial

[2] If more than a trace of carbon is present the run should be discarded.

activity and a corresponding loss of lipoid affinity. Läuger (1) stresses the necessity for two components in the structure of DDT-like compounds necessary for high activity. The first is the presence in the molecule of a toxic component which presumably interferes with some enzyme system in the insect physiology. Para chloro, fluoro and methoxyl substituted phenyl rings seem to be the most active groups for this purpose (1, 3, 11). The second is the presence in the molecule of a lipophilous component which causes an accumulation of the compound in the cell lipoids and thus concentrates the toxic component at its site of action, presumably the nerve lipoids. The trichloromethyl group seems to be the most active for this purpose although the dichloromethyl and cycloprophyl groups also produce high activity (6). Extreme toxicity results only when both components are present in the molecule together. It is interesting that compounds I, V, VI and VII are accumulated, presumably because of their trichloromethyl group, in relatively large amounts in the fat of animals to which they are fed. Compound VIII, containing the trifluoromethyl group, is not accumulated under the same conditions although it is also very fat soluble. If the presence of a single group within the molecule confers lipoid affinity upon it and subsequent

TABLE 2

Analyses of samples of perirenal rat fat

FAT FROM GROUP NO.	FLUORINE	COMPOUND EQUIVALENT
	mgm /1 gm fat	*mgm /gm. fat*
I Control	0.00	
II. 5 mg. NaF/kilo body wt., daily	0.00	
III. 50 mgm compound VIII/kilo body wt., daily	<0.01	<0.05 (compd. VIII)
IV. 50 mgm compound VII/kilo body wt., daily	0.38	3.2 (compd. VII)

storage in the body lipoids then it provides a principle which should have wide application in the synthesis and modification of chemotherapeutic agents.

SUMMARY

The compound 1,1-bis(p-flucrophenyl) 2,2,2-trichloroethane which has insecticidal activity very near that of DDT has been shown to accumulate in the perirenal fat of rats to which it was fed. The compound 1,1-bis(p-chlorophenyl) 2,2,2-trifluoroethane which has little or no insecticidal activity did not accumulate in the perirenal fat of rats under similar conditions. This evidence points rather definitely to a relationship between lipoid affinity and insecticidal activity and as such offers an explanation for the mechanism of action of 1,1-bis(p-chlorophenyl) 2,2,2-trichloroethane and related insecticides as suggested by Läuger.

The significance of these data and other evidence in the literature is discussed.

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THE ACTION OF ALLOXAN, SENECIONINE, SULFADIAZINE, AND THIOURACIL IN THE HAMSTER

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Although the Syrian hamster, *Cricetus auratus*, has proved to be a useful laboratory animal for the study of a variety of infectious diseases, pharmacological studies of the hamster have been few. Recently, Chen, Powell, and Maze (1) have reported investigations upon the response of hamsters to a group of drugs, including those acting on the central nervous system; those acting on the cardiovascular system; those acting on smooth muscle organs; insulin; and sodium cyanide.

It is the purpose of this paper to present information upon the reaction of the hamster to four additional compounds: alloxan monohydrate, senecionine, sulfadiazine, and thiouracil. These substances are known to cause lesions in parenchymatous or endocrine organs of other animals.

PROCEDURES AND RESULTS. These experiments were run simultaneously with those reported previously (1), and the conditions under which the hamsters were kept were identical; that is, the experiments were done during June and July with animals which had been acclimated in air-conditioned quarters for at least a week at temperatures ranging from 26.7° to 28.9°C., and fed on "Purina Laboratory Chow" and kale. These animals weighed between 80 and 122 grams, averaging 100.7 grams at the beginning of the experiment.

Alloxan monohydrate. The discovery by Dunn, Sheehan, and McLetchie (2) that alloxan produces selective necrosis of pancreatic islet cells initiated a large number of investigations. These have been reviewed recently by Joslin (3) and by Chen (4). In our experiments alloxan was administered intravenously to 24 hamsters in single doses ranging from 40 to 200 mg. per kg. Eight animals survived for a week and were then killed with chloroform vapor for histologic examination of the viscera. Six moribund animals were also killed with chloroform in order to facilitate collection of blood samples for glucose determination and to secure fresh tissues for microscopy; one animal was killed on the second day, 4 on the third, and another on the fourth. Pertinent data are included in table 1. The survival period of each animal is given individually even in those groups in which figures are identical.

Terminal blood sugar levels are tabulated for each of 7 animals. No attempt was made to follow the changes in blood sugar levels of individual animals, and determinations were not made on those animals which survived for 7 days. It will be noted that the blood sugar attained very high levels, as contrasted with the mean normal blood sugar of 15 animals which was determined to be 92.5 ± 4.1 mg. per 100 cc. (1).

The livers, kidneys, and adrenals of 23 hamsters, the pancreases of 21, and

the pituitary, heart, and lungs of smaller numbers were examined microscopically. The pancreatic islands of 5 of the 8 hamsters which survived for 7 days were normal. In the other three, minimal injury was manifested in some islands by the presence of two or three beta cells with pyknotic, shrunken nuclei. Necrosis of beta cells had occurred in the islands of all other hamsters, the extent of injury ranging from slight to severe. An example is shown in Figure 1A. The animals that received the largest doses usually showed the greatest injury. Hydrops of beta cells was observed in 4 hamsters.

The kidneys of animals that survived 7 days were normal. The kidneys of those that died because of treatment showed slight to moderate injury as evidenced by cloudy swelling, with fatty metamorphosis in four cases, and necrosis of varying extent of cells of the convoluted tubules of 12 animals. An example is given in figure 1B Fatty metamorphosis of liver cells occurred in many hamsters, the degree in general increasing with the dose of the drug Three animals had moderate or extensive pulmonary edema, and a fourth had atelectasis and edema of one lung The other viscera were not abnormal

TABLE 1

Toxicity of alloxan monohydrate and of senecionine by intravenous injection

COMPOUND	DOSE	NUMBER DIED* / NUMBER USED	$LD_{50} \pm S E$	NECROPSIES After death	NECROPSIES After sacrifice	SURVIVAL	BLOOD SUGAR
	mg per kg		*mg per kg*			*days*	*mg per 100 cc*
Alloxan monohydrate	40	0/3		0	3	7, 7, 7	
	50	1/3		1	2	4, 7, 7	
	62	2/3		0	2† + 1	3, 4, 7	607, 822
	80	1/3		1	2	3, 7, 7	
	100	3/3	65 4 ± 9 8	3	0	3, 3, 4	
	125	3/3		2	1†	3, 3, 3	770
	160	3/3		1	2†	3, 3, 3	573, 633
	200	3/3		2	1†	2, 2, 2	458, 647
Senecionine	50	0/3		0	3	7, 7, 7	
	56	1/3		1	2	3, 7, 7	
	62	1/3	61 1 ± 2 9	1	2	3, 7, 7	
	70	3/3		3	0	2, 2, 3	
	80	3/3		2	0	0, 2, 4	

* The figures for alloxan include 6 moribund animals that were killed instead of being allowed to die spontaneously

† Moribund

Our data on the effect of alloxan on the hamster are not strictly comparable with the data on other animals in the literature Apparently, the LD_{50} has not been determined for other species Many of the hamsters died without our having determined the blood sugar level, and blood sugars of those that survived a week were not tested Inasmuch as the pancreatic islands of 5 of

the survivors were normal, it is probable that the blood sugars were normal; and since the insular lesions in the other 3 survivors were slight, it is doubtful that their blood sugar levels were greatly altered. However, since serial or multiple sections of the pancreas were not made, we cannot be sure that our sections are truly representative, and it must be admitted that these 3 animals may have had mild diabetes. Goldner (5) defines the diabetogenic dose "as the amount of alloxan which in 80% of the animals of a given species will produce sustained hyperglycemia and necrosis of the pancreatic islet cells, but which will not cause observable damage to other organs." He determined the diabetogenic dose for several species to be: Dalmatian hound, 50–100 mg. per kg., intravenously; monkey, 100–150 mg. per kg., intravenously; pigeon, 125–200 mg. per kg., intravenously; cat, 150 mg. per kg., intravenously; rabbit, 100–200 mg. per kg., intravenously; and rat, 200–300 mg. per kg., intraperitoneally.

FIG. 1A 310× Pancreas of a hamster that received 160 mg. per kg. of alloxan and was moribund 72 hours later. The blood sugar had risen to 573 mg. per 100 cc. It should be noted that some island cells persist, but the majority are necrotic and their nuclei have undergone lysis; several pyknotic nuclei are present

B 120× Kidney of the same hamster Groups of convoluted tubules are necrotic and their nuclei are no longer distinguishable

We may be warranted in surmising on the basis of the LD_{50} and the blood sugar levels obtained at the 62 mg. per kg. dose that the diabetogenic dose is probably in the vicinity of 60 mg. per kg. Corroboratory evidence is supplied by the fact that necrosis of renal tubules did not occur in animals that received doses lower than 80 mg. per kg. It is thus evident that of all species for which data are available, the hamster is the most susceptible to alloxan.

Senecionine. The alkaloid senecionine is known to occur in several species of the genus *Senecio* (6), and has already been shown to produce necrosis of the liver in mice (7), rats (8), and monkeys (9). As in previous experiments, senecionine was prepared for administration by dissolving a weighed quantity in an equimolecular amount of hydrochloric acid. Appropriate single doses were injected intravenously with the results presented in table 1. A total of 15 hamsters was used, and necropsy was performed on all but one that died

immediately after injection of a dose of 80 mg. per kg. At the end of a week, all survivors, 7 in number, were killed with chloroform vapor.

The livers of 2 of the 7 survivors appeared normal, and sections of the livers of 2 others revealed loss of a few parenchymal cells from about the portal spaces. The liver of the fifth animal contained many infarcts which involved only one or a few lobules and were heavily infiltrated with leukocytes. The liver of each of the other 2 hamsters contained a single infarct which involved several lobules, and in one of these there was evidence of loss of a few cells from many periportal areas. The kidneys and adrenals of 6 of these animals were examined microscopically and found normal.

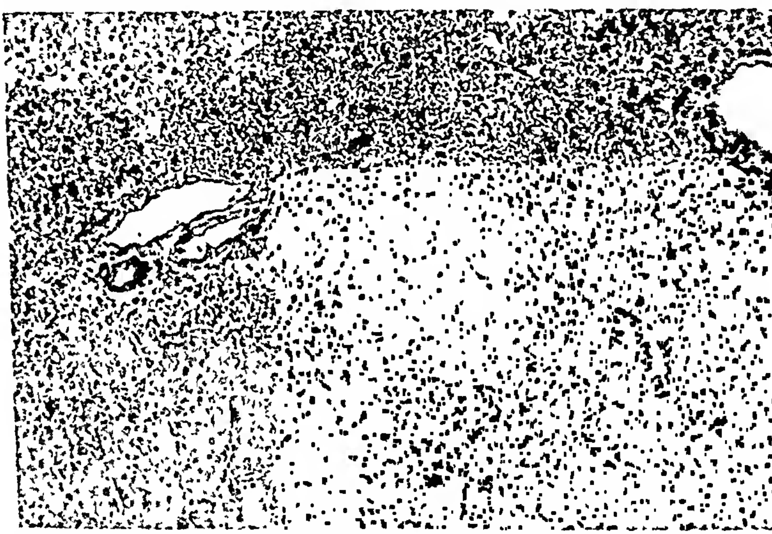

FIG. 2. 120× Liver of hamster that received 62 mg per kg. of senecionine and died 72 hours later. A portal triad lies to the left of the center of the field. There has been much necrosis and hemorrhage, principally about the portal space. The small black dots are erythrocytes. Minute fat vacuoles are present in some liver cells

The livers of the 7 animals that died as a result of treatment all showed necrosis, hemorrhage into cell cords, and sinusoidal congestion. In one liver the lesion occurred in central and midzonal regions; in a second, it involved central and periportal regions with equal frequency; and in the remaining five, it was predominantly or exclusively periportal in distribution. The section in figure 2 is a good representative. Four of these 7 hamsters had some hydrothorax and a few pulmonary petechiae, and one also had slight pulmonary edema. All other viscera appeared normal grossly. Three hearts were studied microscopically and found normal. Kidneys and adrenals of 5 hamsters were sectioned. A focus of cortical sinusoidal congestion was present in one adrenal. There was slight fatty degeneration of the renal convoluted tubules of 1 hamster;

and in the kidneys of 2 other hamsters, necrosis of a few cells of the convoluted tubules had occurred.

A comparison of the reaction of the hamster, monkey, mouse, and rat to senecionine may yield several points of interest. Since the data about rats were not available, a group of 32 were injected intravenously with this alkaloid. The $LD_{50} \pm$ S. E. was found to be 41.2 ± 2.8 mg. per kg. Of the 31 rats examined, the following changes were observed: Ascites, 11; hydrothorax, 12; pulmonary edema, 7; petechiae in the thymus, 8, and in the lungs, 4; hemorrhage into the pancreas and mesentery of the small intestine, 3; slight bleeding into the gastrointestinal tract, 5; and necrosis of the liver with sinusoidal congestion and hemorrhage into cell cords, 22. Necrosis of the livers was all central except that of one, which was periportal and midzonal. Fat was present in the cells of the renal convoluted tubules of 14 animals.

It is thus seen that by comparison of the LD_{50}'s senecionine is equally toxic to mice and hamsters, but more toxic to rats. Necrosis of the liver in hamsters and monkeys is predominantly periportal, but in mice and rats, is predominantly central. Senecionine commonly causes fatty degeneration of the kidneys in monkeys and rats, but rarely does so in hamsters and mice. As with rats and mice, administration of senecionine to hamsters is followd by evidences of endothelial cell injury; that is, development of petechiae, and transudation of fluid from capillaries.

Sulfadiazine. Sulfadiazine was administered to 10 hamsters by incorporating 2 parts of the drug with 98 parts of the food. It may be pointed out that hamsters seem to prefer pellets. It was for this reason that the medicated food was reworked into pellets. At the end of 4 weeks the animals were chloroformed and blood samples were taken. All of them lost weight. The thyroid glands of some hamsters appeared slightly enlarged, and microscopic sections revealed some hypertrophy of the thyroid of each animal. The capillaries were congested, colloid was diminished, and the epithelial cells were appreciably enlarged, with formation of small papillary masses in some acini.

The thoracic and abdominal viscera were normal. No calculi were seen in any part of the urinary tract. The kidneys of 2 hamsters were entirely normal microscopically; there was some leukocytic infiltration beneath the pelvic epithelium of one kidney of a third animal; and in sections of the kidneys of the remaining seven, there was some dilatation of a few small groups of convoluted and collecting tubules. The dilated tubules were empty, and there was no inflammatory reaction about them.

The blood concentration of sulfadiazine in the treated animals varied from 15.5 to 21.5 mg. in free form, and from 0.3 to 3.5 mg. in conjugated form, per 100 cc. Obviously, hamsters do not conjugate sulfadiazine to any great extent. This may explain the lack of injury to the kidneys.

Thiouracil. The independent discovery by Richter and Clisby (10), Kennedy (11), and the MacKenzies (12) of the goitrogenic action of thiourea and its derivatives has stimulated studies of additional compounds. In the short time which has elapsed since Astwood's publication on the treatment of hyperthy-

roidism with thiouracil (13), many observations on this compound have been published.

In our work with hamsters, thiouracil was incorporated in the diet to the extent of 0.1 percent. A total of 10 animals was employed. At the end of 25 to 28 days the animals were killed and their viscera were examined. There was loss of body weight in each case ranging from 6 to 26 grams. The thyroids were congested, but only slightly enlarged, and the other organs appeared normal. Microscopically, there were congestion of capillaries and veins in the thyroids, diminution of colloid, and some enlargement of acinar cells. The colloid in some acini was faintly eosinophilic, and in other acini it was pale and granular. The acinar cells were enlarged and usually of low columnar type, although many were only cuboidal. In some acini the cells formed papillary ingrowths. The nuclei were slightly enlarged and rounded. Figure 3 contrasts the thyroid of a thiouracil-treated hamster with that of a normal animal. The degree of

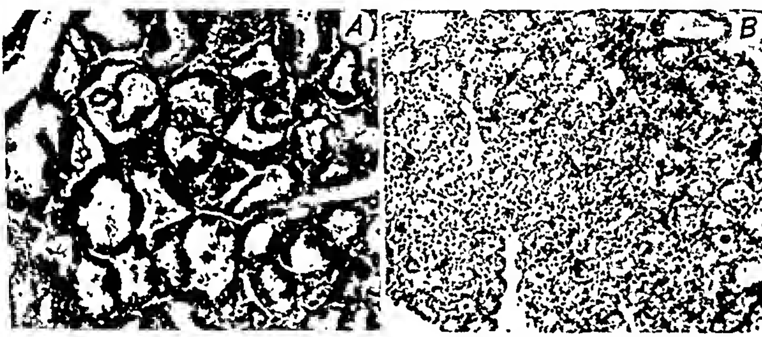

FIG 3.A 120× Thyroid of hamster that received thiouracil in the diet at a level of 0.1% for 25 days Colloid is greatly diminished, the acini are considerably enlarged, and the cells, although only slightly enlarged, form papillary ingrowths into some of the acini.
B. 120× Thyroid of normal hamster Compare with A

hypertrophy of the thyroids of all treated animals was appreciably less than shown by a group of similarly treated rats. However, since the hamsters lost weight, while the rats gained, it is possible that the former did not eat sufficient medicated food to develop the full effects of thiouracil. Unquestionably, the goitrogenic action of thiouracil on the hamster is the same as that on the mouse, rat, guinea pig, rabbit, and dog.

SUMMARY

1. Intravenous administration of appropriate single doses of alloxan to the hamster leads to necrosis of renal tubules, and to injury of the beta cells of the pancreatic islands with development of very high blood sugar levels.

2 Intravenous administration of single doses of senecionine to the hamster results in development of periportal necrosis of the liver.

3. Prolonged administration of sulfadiazine to the hamster does not result

in severe renal injury, probably because the concentration of the conjugated form in the blood is so low. Slight hypertrophy of the thyroid and decrease of its colloid can be demonstrated.

4. Addition of thiouracil to the hamster's diet results in hypertrophy of the thyroid epithelium and diminution of colloid, but the effect seems to be less intense than in the rat.

Acknowledgment. We are indebted to Dr. Richard H. F. Manske, formerly of the Division of Chemistry, National Research Council, Ottawa, Canada, for the supply of senecionine used in this study. We are also indebted to Misses Nila Maze and Marian H. Ellaby and to Mr. Harold M. Worth for invaluable assistance in these experiments.

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METABOLIC STUDIES ON β-CYCLOHEXYLETHYLAMINES[1]

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There has recently been introduced a series of sympathomimetic compounds related to the β-phenylethylamines, but with a cyclohexyl ring replacing the phenyl group (1–4). The relationship between structure and oxidation by amine oxidase has been extensively studied for the β-phenylethylamines by Beyer (5), and the excretion of a number of these compounds by the rat has been the subject of a recent communication from this laboratory (6). Con-

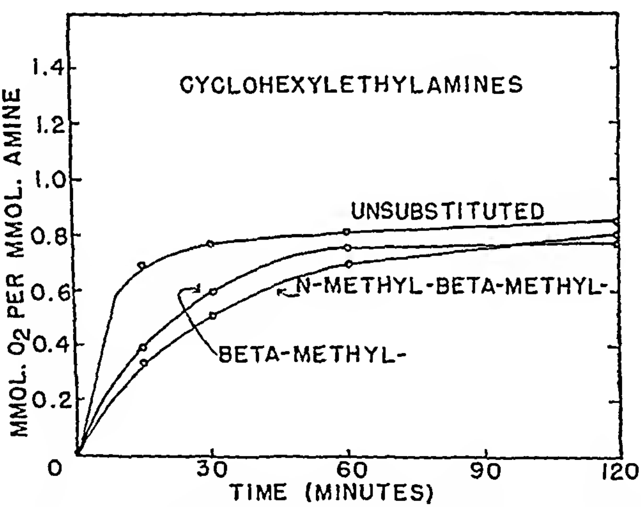

FIG. 1. THE OXIDATION OF β-CYCLOHEXYLETHYLAMINES IN THE PRESENCE OF LIVER AMINE OXIDASE

sequently it appeared worth while to investigate the influence of methyl substitution on the metabolism of the β-cyclohexylethylamines.

METHODS. The procedures used were the same as those previously employed in a study of substituted β-phenylethylamines (6).

The influence of amine oxidase was studied in the conventional Warburg apparatus. The flasks contained 0.0025 mmol. of the amine hydrochloride and 1 cc. of rabbit or guinea pig liver homogenate buffered at pH 7.2. The enzyme preparations were used either

[1] Reported at a meeting of the American Chemical Society, Atlantic City, N. J., April 9–12, 1946.

immediately, with the addition of 0.1 cc. of M/15 sodium cyanide, or after dialysis against distilled water at 0° C. for twenty-four hours.

In excretion tests, 0.05 mmol. of the amine hydrochloride was injected subcutaneously

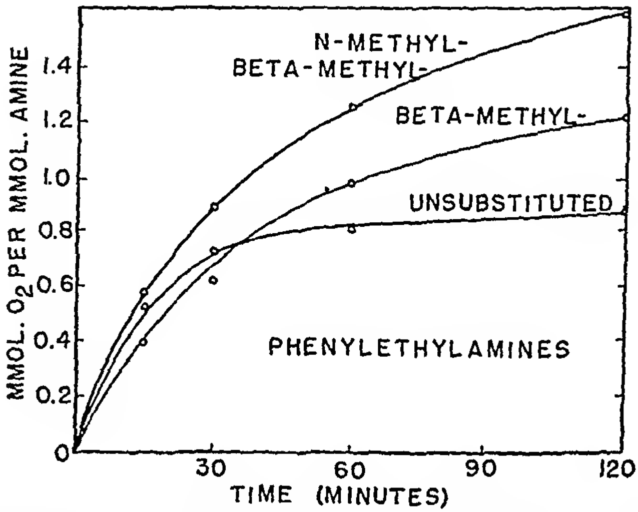

FIG. 2. THE OXIDATION OF β-PHENYLETHYLAMINES IN THE PRESENCE OF LIVER AMINE OXIDASE

TABLE 1

The excretion of β-phenylethylamines and β-cyclohexylethylamines in the urine following subcutaneous administration to rats

COMPOUND	PER CENT OF DOSE EXCRETED IN URINE			
	R = phenyl		R = cyclohexyl	
	Average	Range	Average	Range
$R—CH_2—CH_2—NH_2$	1.7	0.9–3.8	0.2	0– 0.9
$R—CH_2—CH(CH_3)—NH_2$	14.8	9.3–22.6	8.1	4.9–15.3
$R—CH(CH_3)—CH_2—NH_2$	4.2	2.3– 5.8	0.2	0.1– 0.3
$R—CH(CH_3)—CH_2—NH—CH_3$	3.0	0.8– 4.1	0.7	0.6– 0.8

into adult white rats, and the urine was collected for twenty-four hours. Amine excretion was determined by the picric acid colorimetric method of Richter (7). In all cases, an aliquot of the urine was boiled with hydrochloric acid before extraction in order to determine whether any of the compounds were excreted partially in a bound form.

Discussion of results. The compounds[2] included in this investigation were β-cyclohexylethylamine, α-methyl-β-cyclohexylethylamine, β-methyl-β-cyclohexylethylamine, and N-methyl-β-methyl-β-cyclohexylethylamine.

As in the case of the β-phenylethylamines, α-methylsubstitution prevents oxidation by the amine oxidase system. The other three compounds were oxidized to approximately the same extent (fig. 1), but methyl substitution definitely slowed the initial rate of oxidation. This is in distinct contrast to the findings with the β-phenylethylamines (6), where β-methyl and N-methyl substitution markedly increased the total oxygen uptake (fig. 2). No significant differences were observed between results with guinea pig and rabbit livers or with the fresh and dialyzed preparations.

An average of 8% of the dose of α-methyl-β-cyclohexylethyl-amine was found in the urine in 24 hours; slight but probably insignificant increases were observed after boiling the urine with hydrochloric acid. The other compounds were excreted to an extent of less than 1% of the dose, and there was no evidence of conjugation. Table 1 presents these data, together with the results previously reported for the corresponding β-phenylethylamines (6). The extent of destruction is somewhat greater for all of the cyclohexyl derivatives.

SUMMARY

1. α-Methyl substitution of β-cyclohexylethylamine prevents oxidation of the compound in the presence of amine oxidase preparations, and inhibits to a significant extent its destruction following subcutaneous administration to white rats.

2. β-Cyclohexylethylamine and its β-methyl- and N-methyl-β-methyl-derivatives are oxidized by amine oxidase, and are almost completely destroyed by the intact animal.

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[2] The authors are indebted to Dr. S. M. Parmerter of The Wm. S. Merrell Co. for the synthesis of the first compound; to Dr. Glenn E. Ullyot of Smith, Kline & French Co. for supplying the second compound; and to Dr. Bruno Puetzer of Vick Chemical Co. for furnishing the third and fourth compounds.

ON THE RESPONSE OF THE INTESTINE TO SMOOTH MUSCLE STIMULANTS[1]

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The observation that continued exposure of the heart muscle of aplysia to muscarine results in the development of tolerance for this drug formed the basis of the well known potential theory of drug action (1). A similar interpretation has been placed on the findings of Feldberg and Schilf (2) that intestinal smooth muscle can be made insensitive to histamine by constant exposure to large doses of the drug. The observation that isolated intestinal smooth muscle loses it capacity to contract in response to a previously effective dose of histamine following administration of this drug in large doses has also been reported by Barsoum and Gaddum (3). According to Clark this "appears to be one of the clearest cases of a phenomenon that can most simply be interpreted as a potential action" (4). The potential theory of drug action, however, does not enjoy unqualified acceptance as it involves a mechanism that has no counterpart in the well studied phenomena of physical or biological chemistry.

Feldberg and Solandt (5) have recently made the observation that when intestinal muscle is suspended in glucose-free Tyrode solution, it loses progressively its rhythmic activity as well as the capacity to contract in response to repeated administrations of acetylcholine. They ascribe this loss of contractility to the exhaustion of the metabolic reserves from which the muscle derives the energy needed in the process of contraction. It appeared therefore of interest to investigate again the mechanism underlying the loss of contractility induced by large doses of histamine and the experimental findings form the basis of this report.

METHODS. The isolated small intestine of the guinea pig was used in all experiments. The animals were stunned with a blow on the head and bled out. The intestine was washed out thoroughly with warm Tyrode and used immediately. A 3 to 4 cm. segment from the lower third of the ileum was suspended in a bath of 40 cc. capacity. (In some experiments a smaller bath of 4 cc. capacity was used.) The lumen at both ends was left open and the upper end was attached by a thread to a Lovatt Evans frontal lever (6), writing on a slowly moving kymograph. (The magnification was about three times.) The 40 cc. bath in which the strip was immersed was kept oxygenated by a stream of air bubbling slowly through it and maintained at a constant temperature by being immersed in a larger bath. The temperature was controlled within 0.2° C. by an electric thermostat. In all experiments, unless stated, we used a solution of Tyrode freshly prepared from stock solutions, so that the final solution had the following composition: NaCl 0.80%, KCl 0.02%, $CaCl_2$ 0.02%, $MgCl_2$ 0.01%, $NaHCO_3$ 0.1%, $NaHPO_4$ 0.005%. (It may be noted that the K/Ca

[1] This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

ratio of this solution is 1 0 as compared with that of 1 75 for Ringer Locke's solution) After being suspended, the strip was allowed to relax for about 30 minutes and then the drug to be tested, dissolved in 0 4 cc of distilled water, was added to the bath Concentrations are always expressed as the final concentration in contact with the smooth muscle strip The drugs were allowed to remain in contact with the intestinal strip for 45 seconds after which the bath was emptied from the bottom and rinsed twice with Tyrode solution The interval between two subsequent drug administrations was kept constant (4 minutes)

EXPERIMENTAL RESULTS *Experiments with histamine* The contractility of the intestinal strip can be followed for several hours by administering at regular and short intervals an effective dose of a stimulating agent If the dose is kept constant, the response of the intestinal strip after the first two or three contractions becomes stabilized and remains constant for a period of several hours Any change in the response to this dose, which we will hereafter designate

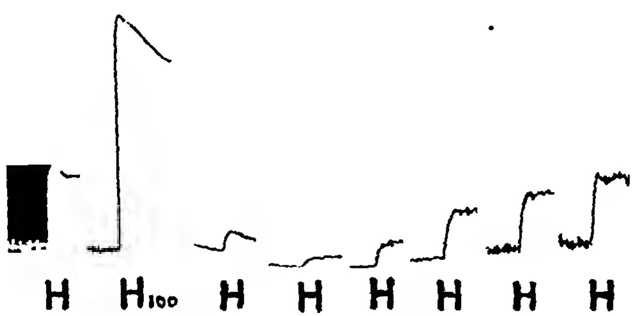

FIG 1 EFFECT OF A LARGE DOSE OF HISTAMINE ON THE RESPONSIVENESS OF INTESTINAL SMOOTH MUSCLE GUINEA PIG ILEUM SUSPENDED IN 40 CC TYRODE
H = histamine 1 200 million (Standard Reference Dose) H_{100} = histamine 1 2 million
Interval between two subsequent doses is 4 minutes

as the *standard reference dose*, must therefore be due to a change in the contractility of the preparation By interpolating a dose of the stimulating agent several times as large as the *standard reference dose*, it is possible to study the effect of this large dose upon the contractility of the intestinal smooth muscle Using histamine we have been able to reproduce the observations of Barsoum and Gaddum that the capacity of the isolated ileum to contract in response to a previously effective dose of histamine is greatly impaired after administration of a large dose of this drug Such an experiment is illustrated in figure 1.

Once the initial level of responsiveness of the preparation to a *standard reference dose* of histamine (1.200 million) had been established, a dose 100 times as large (1.2 million) as the standard was given This resulted, as was to be expected, in a much greater response The *standard reference dose* when then repeated, produced a very small contraction The subsequent *standard reference dose* caused an even smaller response This period of decreased contractility

lasted for about 20 to 30 minutes during which time the response of the strip to the smaller dose of histamine given at regular intervals gradually returned to the initial level.

Almost parallel with the decrease in responsiveness to a stimulating agent there occurred a change in the spontaneous motility of the strip. Immediately following the large contraction produced by histamine in a concentration of 1:2 million, there was an almost complete loss of the spontaneous rhythmic contractions which, however, gradually reappeared in the course of the next 20 to 30 minutes. This effect was not seen in every case as there was often no initial spontaneous rhythmic activity of the muscle strip.

The depression in contractility which followed a large dose of histamine was sometimes so great that the muscle became completely refractory to the drug. The maximum depression usually developed four minutes after the interpolated large dose; in several instances, however, the lowest point was reached later. Such was the case in the experiment reproduced in figure 1. This experiment has been repeated numerous times. As long as the essential features of the experiment were maintained—namely the interpolation of a large dose of histamine in a series of smaller, but effective doses—the results were always the same. Neither changes in the time interval and the number of washings with Tyrode between subsequent applications of histamine nor atropinization resulted in a deviation from the pattern just described.

Experiments with acetylcholine, mecholyl, pilocarpine, and barium chloride. In order to determine the role and specificity of histamine in the production of the loss of contractility just described, a similar experiment was performed using acetylcholine. In this case the *standard reference dose* was 1:10 million. The response of the intestinal strip to this dose became greatly depressed following the interpolation of a dose of acetylcholine 100 times as large. This period of depression lasted for about 20 minutes (fig. 2). Essentially the same result, namely the induction of a period of decreased contractility following a maximal contraction, was obtained by using other stimulating agents such as pilocarpine, barium chloride and mecholyl. It is shown conclusively, therefore, by these experiments that the effect under consideration is not a specific one for histamine.

Another experiment which further illustrates the lack of specificity of the effect described is reproduced in figure 3. In this experiment a series of contractions of the intestinal strip was produced by administration of *standard reference doses* of histamine (1:100 million) while the large interpolated contraction was induced by administration of pilocarpine (1:100,000). This large contraction was followed, as in previous experiments, by a period of decreased responsiveness to the *standard reference dose* of histamine and the period of derceased contractility lasted for about 30 minutes.

Experiments with potassium chloride. The experiments so far described have one feature in common, namely the development of a period of decreased contractility following a large, probably maximal contraction of the intestinal strip produced by different stimulating agents.

This sequence, superficially at least, is the opposite of what takes place in

skeletal muscle. In the skeletal muscle interpolation of a brief tetanus in a series of muscle twitches is followed by a period of increased responsiveness

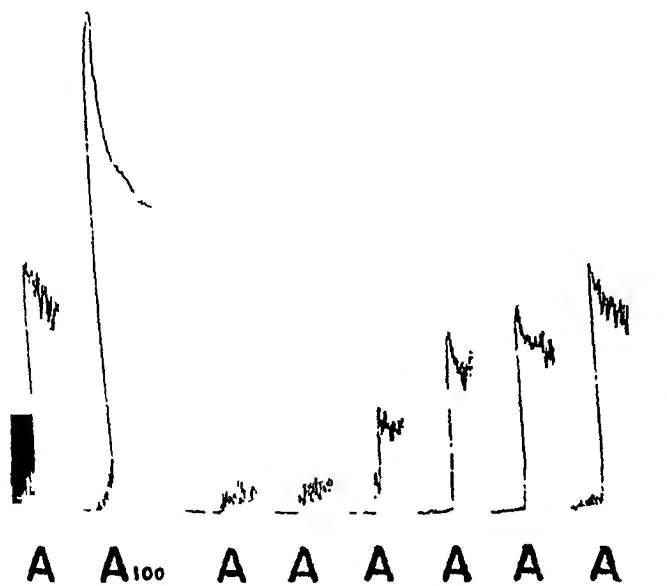

FIG. 2. EFFECT OF A LARGE DOSE OF ACETYLCHOLINE ON THE RESPONSIVENESS OF INTESTINAL SMOOTH MUSCLE. GUINEA PIG ILEUM SUSPENDED IN 40 CC. TYRODE
A = acetylcholine chloride 1:10 million (Standard Reference Dose). A_{100} = acetylcholine chloride 1:100,000. Interval between two subsequent doses is 4 minutes

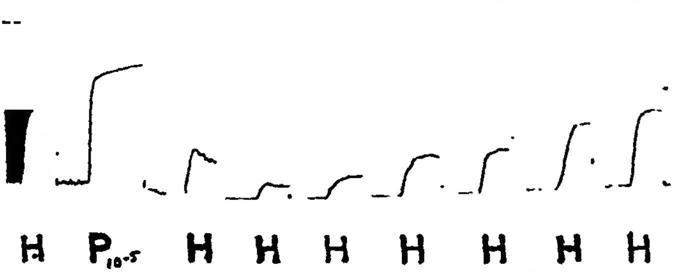

FIG. 3. EFFECT OF A LARGE DOSE OF PILOCARPINE ON THE RESPONSIVENESS OF INTESTINAL SMOOTH MUSCLE. GUINEA PIG ILEUM SUSPENDED IN 40 CC TYRODE
H = histamine 1:100 million (Standard Reference Dose). $P10^{-5}$ = pilocarpine 1:100,000 Interval between two subsequent doses is 4 minutes.

known as "post-tetanic potentiation." This phenomenon which has been thoroughly investigated by different workers (7) has been attributed to an

accumulation of potassium ions outside the muscle fiber (8). In support of this view is the observation that the interarterial injection of minute amounts of potassium chloride results in an increase in the size of the twitches of normal muscles (9). It appeared important therefore to determine what effect a large interpolated contraction produced by potassium chloride might have on the responsiveness of the intestinal smooth muscle to a stimulating agent such as histamine. When such an experiment was performed, there occurred no decrease in the responsiveness of the preparation. As a matter of fact the responses to a *standard reference dose* of histamine (1:100 million) became larger than before the administration of potassium chloride and gradually returned to normal (fig. 4). This, it should be emphasized, is in marked contrast with what happens

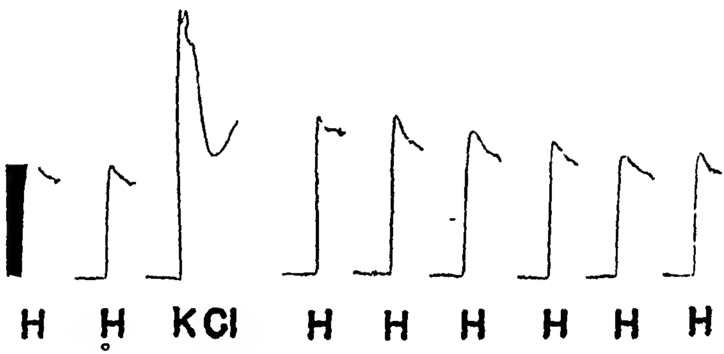

FIG. 4. EFFECT OF A LARGE DOSE OF POTASSIUM CHLORIDE ON THE RESPONSIVENESS OF INTESTINAL SMOOTH MUSCLE. GUINEA PIG ILEUM SUSPENDED IN 40 CC. TYRODE. H = histamine 1:100 million (Standard Reference Dose). KCl final concentration: .22% or eleven times that of Tyrode. Interval between two subsequent doses is 4 minutes.

when a large contraction produced by histamine or other stimulants is interpolated in a series of smaller contractions (figs. 1, 2 and 3).

The dose of potassium needed in this experiment to bring about a maximal contraction of the smooth muscle strip was somewhat large, representing an elevenfold increase in the potassium concentration of Tyrode's solution. Therefore we decided to determine whether a smaller increase in potassium concentration of the perfusion fluid would have a neutralizing effect on the phase of decreased contractility which follows a large stimulating dose of histamine. To perform this experiment the intestinal strip was suspended in a solution containing twice the normal amount of potassium chloride in Tyrode (a solution to which we will refer as K-rich Tyrode). This change to K-rich Tyrode did not result in any immediately obvious change in the activity of the isolated guinea pig intestine, but the response to repeated equal doses of histamine became somewhat larger. Once the response to a *standard reference dose* of

histamine (1:200 million) had become constant, a dose 100 times as large was interpolated with a resultant larger contraction. This was *not* followed by a decrease in the responsiveness of the strip (fig 5). It is clear, therefore, that even a small increase in the potassium concentration of the perfusion fluid, by itself devoid of an obvious effect on the activity of the preparation, is capable of preventing the phase of decreased responsiveness which normally follows a large contraction

DISCUSSION. Essentially two facts emerge from the experiments described: First, the phase of decreased contractility which follows a maximal contraction of intestinal smooth muscle is not a specific phenomenon as it can be induced by histamine, acetylcholine, and a variety of other smooth muscle stimulants.

Second, this phase of decreased contractility is abolished *both by large* doses

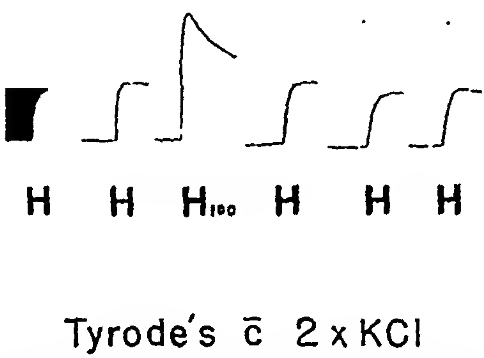

FIG 5 EFFECT OF A LARGE DOSE OF HISTAMINE ON THE RESPONSIVENESS OF INTESTINAL SMOOTH MUSCLE SUSPENDED IN K RICH TYRODE SOLUTION

H = histamine 1 200 million H_{100} = histamine 1 2 million Interval between two subsequent doses is 4 minutes

of potassium, capable of initiating by themselves a contraction of smooth muscle, and *by smaller* doses of potassium, which by themselves have no such effect.

As regards the specificity of the phase of decreased contractility which formed the object of our investigations, our observations differ from those of Barsoum and Gaddum (3) These authors reported that following administration of a large dose of histamine, isolated intestinal smooth muscle exhibited only a slightly diminished response to acetylcholine, barium, etc while it had become insensitive to a previously effective dose of histamine The observations of Barsoum and Gaddum were obtained from experiments on the fowl's caecum but their conclusions about the specificity of this effect for histamine were later extended to include other types of intestinal smooth muscle (10). In view of our observations, it is clear that the reaction of the intestinal smooth muscle of the guinea pig is different from that of the fowl As the guinea pig's ileum is generally used for biological assay of histamine, it appears that the widely

employed practice of regarding the insensitivity to histamine which follows a large dose of this drug as a criterion for its identification in biological materials is not on firm ground and might lead to error.

An explanation of the mechanism of the phase of decreased contractility is not immediately forthcoming. It has been shown by different investigations that the stores of chemical energy available to smooth muscle for the contraction process are rather small. In this respect intestinal smooth muscle is quite different from other types of muscle tissue, such as the heart, which exhibit spontaneous rhythmic activity. The carbohydrate reserves and the spontaneous rhythmic activity of the smooth muscle strip can be depleted by various procedures such as asphyxia of short duration, poisoning with iodoacetate and perfusion with glucose-free solution (5, 11, 12). The heart muscle exhibits much greater resistance to similar treatment. The decreased contractility which formed the object of our experiments, though it develops in the presence of glucose, can be interpreted along lines similar to those suggested by Feldberg and Solandt (5) to explain the loss of contractility observed in glucose-free solutions. The huge energy expenditure which underlies a maximal contraction of smooth muscle might lead to exhaustion of the available stores of chemical energy and thus afford an explanation for the depression of contractility which develops in the isolated guinea pig's intestine after the maximal contraction.

As regards the mechanism whereby potassium neutralizes the phase of decreased contractility only speculation is possible. By the use of muscle homogenates and partially purified enzyme preparations it has been established that potassium plays an important part in the glycolytic esterification of phosphate (13, 14). Boyer, Lardy and Phillips (15) have shown that potassium has an essential and specific role in the transfer of phosphate from 2 phosphopyruvate or 3 phosphoglycerate to the adenylic system. Similarly potassium is essential in the phosphorylation of creatine which accompanies pyruvate oxidation. Calcium ions have been shown by these investigators to be directly antagonistic to the transfer of phosphate to the adenylic system. Our observations could be explained on a similar basis if we are to assume that potassium ions also play such a role in the intact smooth muscle. When the intestinal strip is immersed in solutions whose K/Ca ratio is higher than 1.0 there would occur an increased formation of energy yielding metabolites such as phosphocreatine or adenosine triphosphate. It is well to emphasize, however, that such an explanation is of a hypothetical nature.

SUMMARY

1. The effect of large doses of histamine, acetylcholine, pilocarpine, and potassium chloride on the responsiveness of isolated guinea pig intestine was investigated.

2. Maximal contractions obtained in response to a large dose of histamine, acetylcholine, pilocarpine, barium chloride, and mecholyl were followed by temporary depression of the contractile responsiveness of the intestinal strip.

On the other hand, a maximal contraction in response to large doses of potassium chloride did not result in a decreased contractility of the preparation.

3. A small increase in the K/Ca ratio of the perfusion fluid, such as obtained by doubling the potassium content of Tyrode solution, was sufficient to neutralize the effect of large doses of histamine, acetylcholine, pilocarpine, barium chloride, etc.

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GENERAL SYSTEMIC ACTIONS OF DI-ISOPROPYL FLUOROPHOSPHATE (DFP) IN CATS[1]

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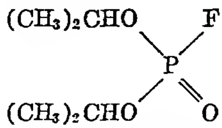
It was discovered by Lange and von Krueger (1) in 1932 that alkyl esters of fluorophosphoric acid gave rise to toxic vapors which produce laryngeal, respiratory, and visual disturbances in fairly low concentrations. In 1941 McCombie and Saunders (2) undertook the synthesis of related compounds which offered promise of usefulness as toxic war gases. In preliminary experiments they found that the di-isopropyl ester fluorophosphate (DFP) provided one of the most potent lethal inhalants. This compound



has been the subject of numerous investigations during the war, described in various special reports. The power of the fluorophosphates to inhibit cholinesterase, described by Mackworth (3), has attracted most attention.

The present paper deals with the general systemic actions of DFP by intravenous injection in cats.

Toxicity. The potency of several samples of DFP was found to vary. The data relating to the range of fatal doses have been charted in figure 1. The material designated as Sample A includes two specimens of DFP obtained from University of Chicago in September and November, 1943. Shortly after they were received they were sub-divided and sealed in small pyrex glass ampoules. One of these ampoules was opened on each experimental day, diluted with distilled water to make a 1% solution, and the desired quantity injected rapidly intravenously. The mortality rates after doses of from 1 to 10 mg. per Kg. are represented in the figure. From these data the LD50 of Sample A was calculated to be 3.6 mg. per Kg. The results show considerable irregularity, partly due to deterioration, since at the time these experiments were carried out, the fact of fairly rapid deterioration was not appreciated. One of the ampoules when opened, and although chilled to 0°C., was under high pressure indicating that some change had occurred. It was also found that diluted solutions show appre-

[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College

[2] This study is part of a coöperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

ciable reduction in potency during the course of the day. Adkins and Wilde (4) showed that the compound undergoes half-hydrolysis in 16 hours in aqueous solution. While these factors reduced the absolute accuracy of the LD50 for Sample A, the value is useful from the standpoint of the fact that during the time in which it was obtained, other studies relating to protection against DFP were in progress with the same materials.

The material designated Sample B was received from the Biochemical Division of the Chemical Warfare Service at Edgewood in June, 1944. Its potency was

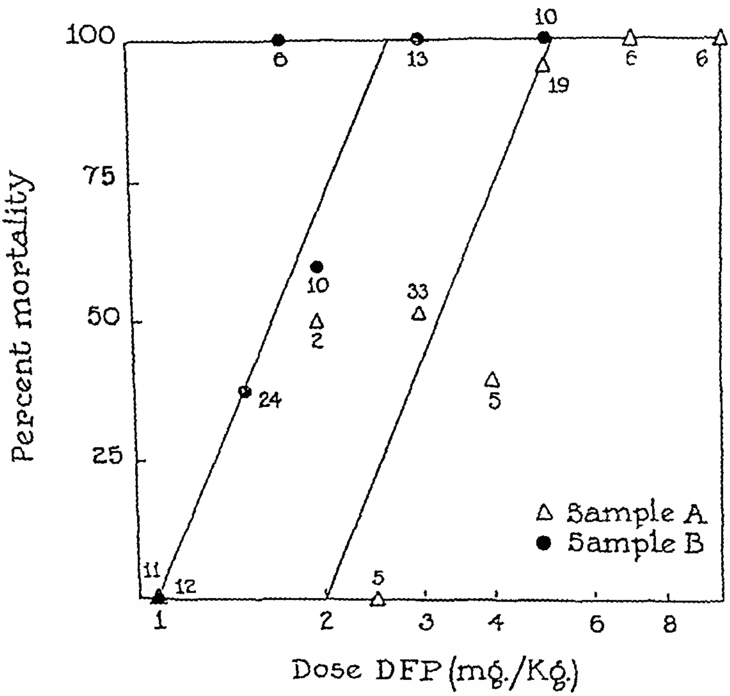


FIG. 1. LOG (DOSE)—MORTALITY CURVES FROM WHICH THE LD50 DOSE WAS DETERMINED FOR TWO SAMPLES OF DFP
Figures at points represent number of cats for each dose

about twice that of Sample A, the LD50 being approximately 1.7 mg. per Kg. (fig. 1). The results with this specimen show much less variation because at this time we were aware of the necessity of using the material shortly after dilution.

Account was taken of the difference in the potency of Samples A and B in the interpretation of the influence of other agents on the toxicity of DFP.

The study by Horton, Koelle, McNamara, and Pratt (5), showed that the susceptibility of the cat occupies a position between the much more susceptible rabbit and more tolerant dog. The report of Smith and Gates (6) indicated that

by inhalation of the vapor the monkey was much more susceptible than the rabbit.

Symptoms after intravenous injection. These were observed in more than 200 cats, in 81 of which they were studied in some detail. DFP was injected in a 1 per cent aqueous solution in a single dose in amounts varying from 1 to 8 mg. per Kg. The symptoms were motor unrest, signs of apprehension, muscular twitching which usually began in the head and neck region, head or body tremor, ataxia, weakness, pilomotor stimulation, respiratory stimulation (panting) associated with labored respiration, salivation and defecation or diarrhea. There was no vomiting. There was sluggish response to a painful stimulus. The foregoing symptoms progressed, the respiratory difficulty increased, and the unrest with thrashing about became extreme. Death was sometimes preceded by typical camphor-like convulsions. After the first few hours the motor unrest ceased, and in most cases the outstanding symptoms in advanced poisoning were prostration with shallow or labored respiration.

TABLE 1

Duration of symptoms in fatal cases (unprotected cats)

NO. OF CATS	DOSE	DURATION OF SYMPTOMS	
		Range	Average
	mg./Kg.		
1	2.0 A	27 min.	
17	3.0 A	12 min.–1 day	3.5 hours
2	4.0 A	51 min.–23 hours	10.2 hours
18	5.0 A	6 min.–2.3 hours	32 minutes
3	6.0 A	2.5–6 days	3.7 days
6	7.0 A	4–17 min.	9 min.
3	8.0 A	24 min.–14.5 hours	5.6 hours

There was a latent period after the injection before symptoms appeared. It varied from about 1–18 minutes after the doses used. There were marked individual differences. For example, a 3-mg. dose produced effects in 2 minutes in one cat; in another only after 18 minutes. In general the latent period was longer the smaller the dose.

The course of the poisoning varied and terminated in one of three ways; death, fairly prompt and apparently complete recovery, or protracted symptoms.

The data concerning the duration of the effects in normal cats which succumbed are summarized in table 1. There were marked individual variations. For example, one cat succumbed to 3 mg. per Kg. in 12 minutes whereas in another, the course ran as long as 24 hours. The largest doses usually caused death in a matter of a few minutes, whereas in the case of the smaller fatal doses the course was often longer terminating fatally after a period of many hours, with profound prostration and a combination of respiratory and circulatory failure. The relation between the size of the dose and the duration of poisoning is clearly seen in the results with the fairly large groups which received 3 and 5 mg.

respectively. The 5-mg. group died in an average of 32 minutes, while the 3-mg. group took on the average of 7 times as long (3.5 hours). There were a few fatal cases in which the course was prolonged to several days.

The data concerning the duration of effects in normal cats which survived are summarized in table 2. After doses of 2.5 mg. per Kg. or more, symptoms of poisoning usually persisted for several days. Again, there were marked individual variations in the duration of the symptoms, one animal recovering completely after one day and another taking more than 17 days after a similar dose.

One cat not included in the table, which received a total of 6 mg. in 3 doses over a period of 2 weeks, after the acute symptoms subsided, developed weakness, ataxia, and pilomotor stimulation, which were still present more than 5 months later. This appeared to be a permanent injury by DFP.

Symptoms of this nature and of unusually long duration were fairly frequent in another series of animals which by reason of protection by other agents were

TABLE 2

Duration of symptoms in survivors (unprotected cats)

NO. OF CATS	DOSE	DURATION OF SYMPTOMS	
		Range	Average
	mg./Kg.		
1	1.0 A		
1	2.0 A	1 day	
6	2.5 A	1–6 days	3.3 days
15	3.0 A	1–17+ days	4.7+ days
3	4.0 A	3–4 days	3.6 days
5	5.0 A	6 hours–4 days	1.8 days

enabled to survive much larger doses of DFP. The results are summarized in tables 3 and 4.

In table 3, which shows the fatal cases, the duration of symptoms was longer than in the unprotected animals (see table 1), and lasted as long as 11 days in one and 15 days in another.

The most striking results are seen in table 4. Whereas unprotected animals never survived doses above 5 mg., survivals were common with doses of 7 and 10 mg. of DFP among animals which received atropine, the combination of atropine with magnesium, or physostigmine. In these animals, after acute symptoms subsided, nicotine-like actions appeared, intense muscular weakness, ataxia, and muscular twitching, and persisted for periods of a week or two, in some cases for several months. In the case of the animals protected with physostigmine there was a delay in the development of the chronic symptoms. Nearly all appeared normal on the day following the DFP injection, and only after a period of from 2 to 23 days (average 11½) did ataxia appear in about half the animals. This symptom persisted as long as the animals were kept under observation (5 to 28 days).

The fact that symptoms disappear in about 3 to 4 days after a dose of 4 mg. of DFP but persist for an average of about 16 days after a dose of 7 mg. favors the assumption that the duration of the effects of DFP is only partly dependent on the elimination of the drug. It suggests that DFP in large doses produces an injury of nicotine-like nature from which animals do not recover quickly, and the indications are that in some the injury may be permanent.

The effects of DFP are intensified by physical stimulation. This is especially noticeable during the period of recovery when the animal seems to be normal

TABLE 3

Duration of symptoms in fatal cases (protected cats)

NO. OF CATS	DOSE	PROTECTIVE AGENTS	DURATION OF SYMPTOMS	
			Range	Average
	mg./Kg.			
12	5.0 A	Atropine	19 min.–2 days	1.2 days
8	7.0 A	Atropine	10 min.–8 days	1.2 days
4	10.0 A	Atropine	9–20 min.	15.2 min.
7	7.0 A	Atropine-magnesium	12 min.–15 days	3.4 days
4	10.0 A	Atropine-magnesium	27 min.–1.7 hr.	1.4 hours
1	7.0 B	Physostigmine	11 days	

TABLE 4

Duration of symptoms in survivors (protected cats)

NO. OF CATS	DOSE	PROTECTIVE AGENTS	DURATION OF SYMPTOMS	
			Range	Average
	mg./Kg.			
29	3.0 A	Atropine	0.5–12 days	8.6 days
7	5.0 A	Atropine	7–61 days	15 days
2	7.0 A	Atropine	6–27 days	16.5 days
6	5.0 A	Atropine-magnesium	0.5–8 days	3.7 days
4	7.0 A	Atropine-magnesium	8–14+ days	10.5+ days
3	10.0 A	Atropine-magnesium	9–10 days	9.3 days
1	3.0 B	Physostigmine	12 hours	
17	7.0 B	Physostigmine	4 hours–50+ days	17+ days

when it sits quietly. At this time, if it is excited or roughly handled, it may develop muscular twitching, ataxia and sometimes complete loss of muscular power. The effect of stimulation is also seen in the pupillary reaction. In this case the pupil may be normal until after a period of exposure to a bright light when a miotic action appears which persists after the animal is placed in dim light. This is probably due to the diminished capacity for the disposal of acetycholine in the case in which the supply of cholinesterase has been reduced by DFP.

The duration of action of DFP was studied in a series of 47 cats which received the compound intravenously in repeated doses according to various schedules of intervals. These results show that the cat does not completely recover within

24 hours from the effects of one-third of an LD50. Such a dose (1 mg. per Kg.) was given daily in each of 3 cats. The early doses produced no visible effects but soon weakness and tremors appeared which progressed until the animals died after from 9 to 13 daily doses, or a total of 9 to 13 mg. per Kg. There was undoubtedly some recovery from the effects of the single doses within 24 hours because, by this method of administration, the animals succumbed only after receiving a total of from 3 to 4 times the LD50.

In the case of the 3 animals in which the daily dose was doubled, as might be expected, the total dose which proved fatal was smaller (4 to 8 mg. per Kg., 2 to 4 daily doses). There is probably involved in these experiments the phenomenon of sensitization of the animal by previous doses rather than accumulation of the drug. There is no indication that previous doses of DFP protect against subsequent ones. The effects of DFP are, therefore, in this respect unlike those of physostigmine. The absence of protection against DFP by previous doses was observed in the case of each of 3 cats which received large doses of DFP (7, 10, 10 mg. per Kg. respectively). They survived these doses as the result of protection by physostigmine. They were free of symptoms within 8 to 11 hours after the DFP. The repetition of the dose of DFP together with the protective dose of physostigmine in from 2 to 29 days proved fatal in each case. It is clear, therefore, that the first dose of DFP had not diminished but apparently increased the sensitivity of the animal to the subsequent doses.

Circulation. The effect of DFP by intravenous injection on the blood pressure was studied in 10 experiments on cats. The essentials of the results are summarized in table 5. "Dial" anesthesia (0.5 cc. per Kg. intraperitoneally) was used in most cases; "nembutal" (30 mg. per Kg. intravenously) in one; and procaine (1% solution) in another for local anesthesia. The DFP was given intravenously in single doses of 0.01 to 2.0 mg. per Kg. and in total doses of from 0.4 to 8.0 mg. per Kg. over periods of from 0.5 to about 4 hours. The behavior of the blood pressure was observed during periods of from 1 to 4 hours. It may be noted that the foregoing doses proved fatal in only 2 animals during the period of observation. The remaining animals, after the periods of study indicated, were used for other purposes and sacrificed. In the two fatal cases there was no abrupt change in the blood pressure, but a gradual fall to zero; in one case within nearly 2 hours after the second dose (total 3.0 mg. per Kg.); in the other case there was a fleeting drop from 166 to 88 after the first dose of 1.0 mg. with prompt recovery, followed by progressive fall of the blood pressure to zero over a period of about 1.25 hours after the final dose of 2.0 mg (total 3.0 mg. per Kg.). The occasional fairly sharp drop of the blood pressure which lasted only a few minutes may be a non-specific injection effect The results of these experiments indicate that DFP does not produce any early significant direct effects on the blood pressure in cats The pressure was well maintained over a period of several hours even in animals which were severely poisoned. Late in the course of poisoning the blood pressure showed a progressive decline. Whether this was the result of some delayed direct action of the drug or whether it was secondary to other factors, such, for example, as the deterioration of respiration, is uncertain.

TABLE 5

Effect of intravenous DFP on blood pressure in cats

CAT NO	INTERVAL BETWEEN DOSES	DOSE	BLOOD PRESSURE*	PERIOD OF OBSERVATION	FINAL BLOOD PRESSURE	REMARKS
	min	*mg/Kg*	*mm Hg*	*min*		
1	0	0.2	194–170	69	170	E C G recorded
	47	0.2	170–170			
2	0	0.2	188–188	100	170	E C G recorded
	7	0.8	176–182			
	19	3.5	176–182			
	1	3.5	182–188			
3	0	1.0	170–170	140	0	Procaine anesthesia Gradual decline of BP
	30	2.0	160– 0			
4	0	1.0	122–122	160	104	Vagi sectioned
	78	2.0	128–122			
	49	2.0	102– 94			
5	0	1.0	134–128	151	88	Vagi sectioned B.P. began to fall in last 30 minutes
	72	2.0	140–134			
6	0	1.0	166– 88–180	145	0	Vagi sectioned
	48	1.0	136–130			
	19	2.0	154– 0			
7	0	1.0	178–172	233	172	Vagi sectioned
	18	1.0	160–160			
	9	2.0	148–148			
	116	2.0	160–118			
	69	2.0	160–124–160			
12	0	0.1	210–170–200	266	175	
	37	0.5	190–190			
	34	1.0	190–185			
	86	1.0	180–180			
	34	2.0	200–200			
	29	2.0	200–160–200			
13	0	0.5	150–150	152	140	Adrenalectomized
	41	0.2	145–145			
	19	0.3	125–125			
	43	0.5	120–120			
	15	0.5	125–125			
	13	1.0	135–130			
	18	2.0	135–130			
16	0	0.5	115–120	124	100	Nembutal anesthesia Adrenalectomized. Cord transected (cervical)
	18	0.5	124–100–124			
	54	1.0	92– 92			

"Dial" anesthesia except where otherwise indicated.

* First figure is level just before injection, subsequent figures indicate maximum changes

Sample "A" of DFP used in these experiments

The effect of DFP on the electrocardiogram was observed in 2 animals (Cat 1 and 2 of table 5), after a total dose of 0.4 mg. per Kg. in one case, and 1.0 mg. in the other case. These doses produced no electrocardiographic changes.

DFP caused slowing of the heart rate. This was seen only during the effect of doses which caused severe poisoning. No effect on the heart rate was observed with doses lower than 2 mg. per Kg. The slowing of the heart was more apt to take place after doses of 4 mg. or larger. The slowing of the heart rate developed

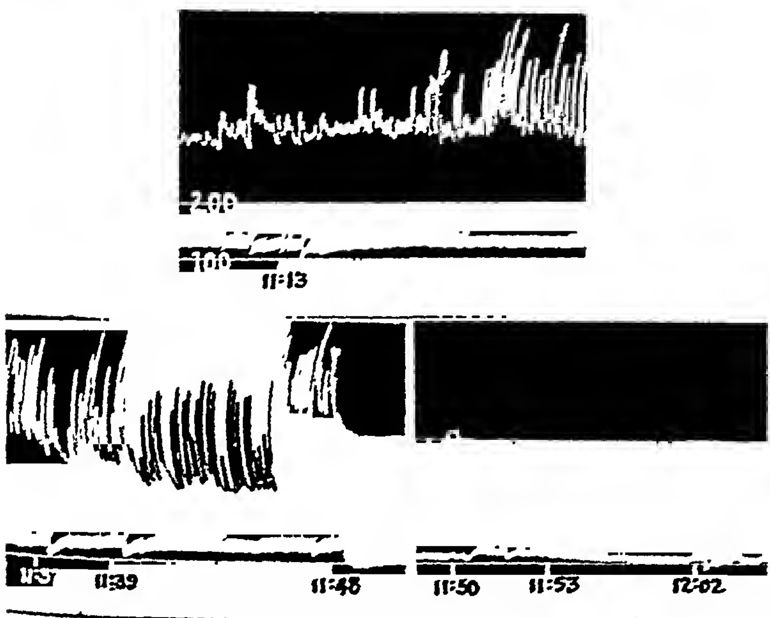


FIG. 2. RECORD OF CONTRACTION OF SMALL INTESTINE IN THE CAT SHOWING INCREASED ACTIVITY FOLLOWING DFP AND ITS ABOLITION BY ATROPINE

11:13 DFP 2 mg. per Kg. intravenously.
11:37 Left vagus cut.
11:39 Right vagus cut.
11:48 Atropine sulfate 1 mg. per Kg. intravenously.
11:50 Left vagus stimulated.
11:53 Right vagus stimulated.
12:02 DFP 2 mg. per Kg. intravenously.

gradually in much the same way as the other signs of poisoning and was most marked during the period when the systemic symptoms were most pronounced. The heart rate sometimes declined to levels of about 70 a minute, which is fairly slow for the cat, under these conditions. The slowing of the heart did not occur in all animals, and while it was apt to be present during advanced poisoning by lethal doses, the heart rate was also sometimes rapid in such cases.

The heart rate and blood pressure changes did not follow any consistent rel-

TABLE 5

Effect of intravenous DFP on blood pressure in cats

CAT NO.	INTERVAL BETWEEN DOSES	DOSE	BLOOD PRESSURE*	PERIOD OF OBSERVATION	FINAL BLOOD PRESSURE	REMARKS
	min.	*mg./Kg*	*mm. Hg*	*min.*		
1	0	0.2	194–170	69	170	E.C.G. recorded
	47	0.2	170–170			
2	0	0.2	188–188	100	170	E.C.G. recorded
	7	0.8	176–182			
	19	3.5	176–182			
	1	3.5	182–188			
3	0	1.0	170–170	140	0	Procaine anesthesia. Gradual decline of BP
	30	2.0	160– 0			
4	0	1.0	122–122	160	104	Vagi sectioned
	78	2.0	128–122			
	49	2.0	102– 94			
5	0	1.0	134–128	151	88	Vagi sectioned. B.P. began to fall in last 30 minutes
	72	2.0	140–134			
6	0	1.0	166– 88–180	145	0	Vagi sectioned
	48	1.0	136–130			
	19	2.0	154– 0			
7	0	1.0	178–172	233	172	Vagi sectioned
	18	1.0	160–160			
	9	2.0	148–148			
	116	2.0	160–148			
	69	2.0	160–124–160			
12	0	0.1	210–170–200	266	175	
	37	0.5	190–190			
	34	1.0	190–185			
	86	1.0	180–180			
	34	2.0	200–200			
	29	2.0	200–160–200			
13	0	0.5	150–150	152	140	Adrenalectomized
	41	0.2	145–145			
	19	0.3	125–125			
	43	0.5	120–120			
	15	0.5	125–125			
	13	1.0	135–130			
	18	2.0	135–130			
16	0	0.5	115–120	124	100	Nembutal anesthesia. Adrenalectomized. Cord transected (cervical)
	18	0.5	124–100–124			
	54	1.0	92– 92			

"Dial" anesthesia except where otherwise indicated.

* First figure is level just before injection; subsequent figures indicate maximum changes.

Sample "A" of DFP used in these experiments.

The effect of DFP on the electrocardiogram was observed in 2 animals (Cat 1 and 2 of table 5), after a total dose of 0.4 mg. per Kg. in one case, and 1.0 mg. in the other case. These doses produced no electrocardiographic changes.

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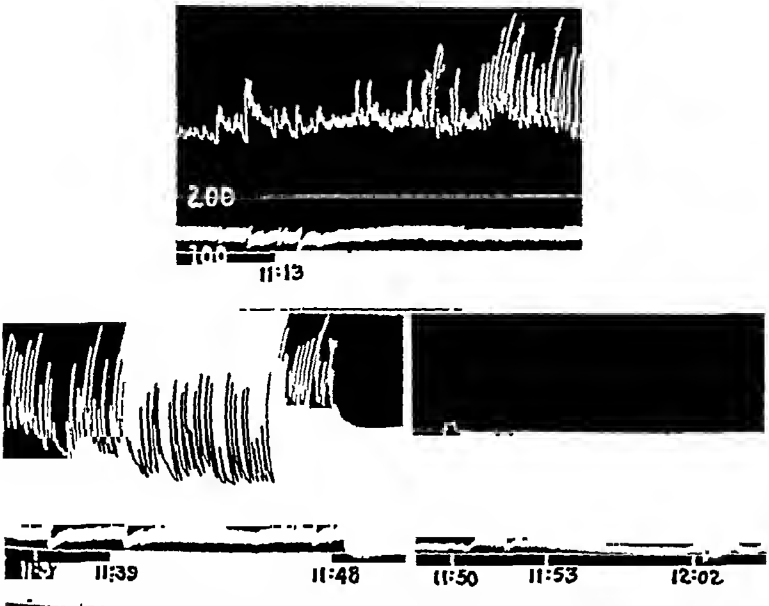

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The heart rate and blood pressure changes did not follow any consistent rel-

tionship, indicating that in the presence of the bradycardia some compensatory action must have been at work to prevent a fall in pressure.

The intravenous injection of 1.0 mg. per Kg. of atropine sulfate after a total of 6.0 mg. of DFP (Cat 7) caused moderate acceleration of the heart rate with slight rise of the blood pressure after a momentary decline. In one case (Cat 4, fig. 2), the atropine caused an abrupt fall of the blood pressure in association with the relaxation of the intestinal tract.

Bilateral cervical vagotomy failed to produce any significant acceleration or change of the blood pressure which was progressively falling after 4.0 mg. of DFP (Cat 6).

Stimulation of the peripheral end of the right severed vagus after a total of

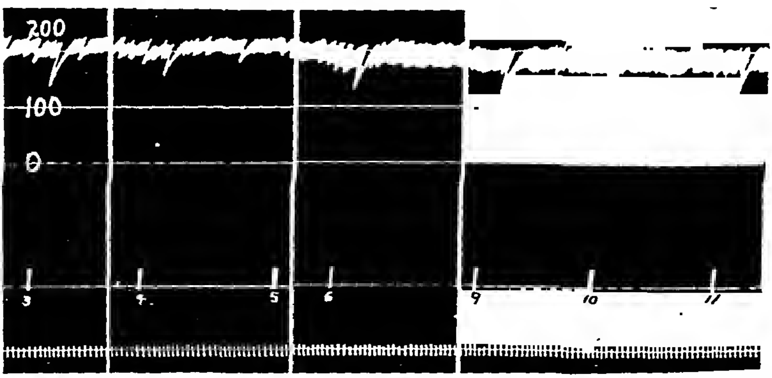

FIG. 3. BLOOD PRESSURE CURVE SHOWING INCREASED SENSITIVITY TO ACETYLCHOLINE AFTER DFP

"Dial" 0.5 cc. per Kg. intraperitoneally.
3—Acetylcholine 0.0001 mg. per Kg. intravenously.
4—Acetylcholine 0.000025 mg. per Kg. intravenously.
5—DFP 0.1 mg. per Kg. intravenously.
6—Acetylcholine 0.00001 mg. per Kg. intravenously.
9—Acetylcholine 0.00001 mg. per Kg. intravenously.
10—DFP 0 5 mg. per Kg. intravenously.
11—Acetylcholine 0.00001 mg. per Kg. intravenously.

4.0 mg. of DFP resulted in fairly typical slowing and fall of the blood pressure (Cat 7).

The intravenous injection of 0.01 mg. per Kg. of epinephrine after 4.0 mg. of DFP (Cat 7) produced the typical rise of the heart rate and blood pressure.

The response of the blood pressure to acetycholine was tested in each of two cats after the administration of DFP. Both showed that DFP markedly sensitizes the blood pressure response to acetylcholine. This sensitization requires very small doses of DFP, as little as 0.02 mg. per Kg., one-hundredth of the amount necessary to produce visible systemic effects in the cat. The sensitization appears within a minute and is fairly persistent, showing no diminution after 30 minutes. The sensitivity of the response to acetylcholine may

increase ten-fold. The change reaches a ceiling after very small doses of DFP as shown by the fact that after a dose of 0.1 mg. per Kg. in one experiment in which the response was sensitized, the administration of doses of DFP up to a total of 7 mg. per Kg. failed to increase the sensitivity further. A typical example is shown in figure 3. There were 19 injections of acetylcholine, mostly of 0.01 microgram per Kg. intravenously at various intervals before and after each of 6 doses of DFP (total 6.6 mg. per Kg.). It may be noted that the dose of 0.025 microgram per Kg. in the control caused a smaller decline of the blood pressure than the 0.01 microgram after DFP, and that larger doses of DFP failed to intensify the response to the subsequent doses of acetylcholine.

Eye. In the normal unanesthetized cat the intravenous injection of DFP produced no significant pupillary changes. The pupil continued to react to light, but as mentioned in the previous section, there was a tendency for the pupil to remain constricted when the animal was put in the dark after exposure to a bright light.

The effect of local instillation of DFP was studied in each of 4 cats, the results of which are shown in table 6. The conjuctival instillation of one drop of

TABLE 6

Local action of DFP on eye

CAT NO.	DFP (INSTILLED LOCALLY)	DURATION OF MIOSIS
	mg	
8	0.6	2½ months
21	0 06	2 months
22	0 006	10 days
29	0.0006	0

solutions varying from 0.001 to 1.0 per cent produced no local irritation in the eye. A dose of 0.0006 mg. was ineffectual, while doses of from 0.006 to 0.6 mg. produced intense miosis which appeared in from 5 to 30 minutes. Some reaction to light remained even after the larger doses when the pupil was reduced to a slit. The miosis persisted for a period of from 10 days to more than 2 months. A phenomenon having the same significance as the one mentioned in connection with the systemic injection of DFP was observed after its local application in the eye, namely, that during the period of recovery, when, in the treated eye, the pupil was of the same size as in the untreated one, a difference between the two pupils could be established by flashing a bright light on the eyes.

The long persistence of the local miotic action suggests some kind of irreversible change similar to that previously discussed in relation to the long lasting effects of the large systemic doses of DFP.

Neuromuscular transmission. In poisoning by DFP, impairment of neuromuscular transmission was observed. It appeared to take place only in the most advanced stage of poisoning when the blood pressure was at very low levels. It is not yet possible to state whether this was a specific action or secondary to

other factors. The changes were observed in each of three cats during "Dial" anesthesia (0.5 cc. per Kg. intraperitoneally). Single doses of 1 to 2 mg. per Kg. were given intravenously at intervals of from 10 to 45 minutes and total doses of from 4 to 13 mg. per Kg. The contraction of the gastrocnemius muscle of the cat was recorded with the isometric technic, the nerve and muscle being stimulated alternately with the Harvard inductorium with tetanic stimuli of 1 second duration at intervals of 50 seconds. The force of the contraction of the muscle when stimulated directly was well sustained from beginning to end. In the terminal 15 to 30 minutes, however, the tension in the muscle during stimulation of the nerve fell off fairly abruptly. The blood pressure in all three animals at this time was very low, 40, 40 and 70 mm. It had been low for a considerable period before the neuromuscular transmission began to fail. The suggestion was obtained in one experiment that anoxia was not the cause of the impaired transmission since effective artificial respiration sufficient to bring about a contraction of the pupil which had become dilated by asphyxia, failed to reverse the downward course of the neuromuscular transmission.

Neuromuscular transmission was tested in each of two cats with continuing symptoms of DFP poisoning 6 to 10 days after the dose was given. These animals looked normal while at rest but when stimulated to activity, after running a few yards they sprawled out on the floor and were unable to stand until they had rested about 5 minutes. With the technic described above, tension records were obtained from the gastrocnemius in response to brief tetanic stimulai applied to the sciatic nerve. In both cats the contractions fell off rapidly during stimulation, in contrast to the well sustained tension of the controls.

Gastro-intestinal tract. It has already been mentioned that the normal unanesthetized cat responds to an intravenous dose of DFP with defecation or diarrhea. We have observed no instance of vomiting. After a fatal dose, direct inspection of the gut showed various degrees of constriction. One animal receiving a large dose had pale, rope-like intestines with almost complete obliteration of the lumen, from just below the stomach to and including the sigmoid.

The behavior of the gut was studied in each of 9 cats in which a balloon was inserted into the duodenum through an opening in the stomach and the contractions recorded by means of a tambour. The animals were anesthetized with "Dial" (0.5 cc. per Kg. intraperitoneally). The DFP was injected intravenously. The single doses varied from 0.1 to 2.0 mg. per Kg. The lowest effective dose was 1.0 mg. per Kg. The response of the gut in these experiments substantiated the observations made by direct inspection. There was an increase in the tone, the rate, and amplitude of contractions. There was a latent period which varied from 1 to 14 minutes. Previous ineffectual doses shortened the latent period. The effect on the gut lasted several hours.

In three experiments, the effect of DFP on the gut was completely abolished by atropine sulfate in an intravenous dose of 1.0 mg. per Kg. It was not found possible to reinstate the effect by additional DFP but the failure to observe mutual antagonism may be a matter of dosage. Bilateral vagotomy reduced the action of DFP on the gut for less than 5 minutes in 2 cases, but was without influence

in two others (DFP total doses of 1 to 4 mg. per Kg.). A typical experiment is shown in figure 2.

In two experiments, epinephrine hydrochloride 0.01 mg. per Kg. was given intravenously. It suspended the effect of DFP on the gut for a period of about 4 minutes.

Blood and blood metabolites. The effect of DFP on the blood and on renal functions as shown by blood metabolites was studied in each of 6 cats. Each received a dose of 7 mg. of DFP per Kg. intravenously and survival was insured by the administration of 10 mg. of atropine sulfate and 0.5 Gm. of magnesium

TABLE 7

The effect of DFP on blood and blood metabolites

CAT NO.	DATE	Hb	R.B.C.	W.B.C.	N.P.N.	N.P.N.
		Gm. %	*millions/mm³*	*thousands/mm³*	*mg./100 cc.*	*mg./100 cc.*
227	2/23/44	13.8	8.4	17.5	47	1.2
	2/26/44	13.5	9.6	27.6	50	1.1
	3/ 6/44	12.5	7.2	22.2	60	1.1
229	2/23/44	12.5	6.2	14.9	54	1.5
	2/24/44	14.8	7.8	31.8	77	1.45
	2/28/44	12.0	7.4	38.4	63	1.45
230	2/23/44	11	5.4	5 3	51	1.5
	2/26/44	14.2	8.0	6 3	57.5	1.5
	3/ 6/44	11.5	6.7	18.1	41.5	1.45
231	2/23/44	14 3	7.6	14.4	61.5	1.1
	2/24/44	14 8	8.1	26.0	73	1.1
	2/28/44	14 0	7.7	6.0	83	1.3
233	3/ 2/44	10 5	6.7	10.7	55	0.9
	3/ 4/44	9 8	5 9	12.2	40	1.2
	3/11/44	9 2	4.1	24.0	40	1.1
234	3/ 2/44	12 8	7 9	17 0	59	1 1
	3/ 4/44	13 0	7.0	15 0	43	1.1
	3/ 6/44	12 0	7.3	10 9	44.5	1.1

sulfate per Kg. intramuscularly. A control value for the following was obtained: R.B.C., Hb., W.B.C., N.P.N., and creatinine. These were repeated twice during the period of observation which lasted from 4 to 11 days. The results are summarized in table 7. They show that the large doses of DFP were without significant effect on the blood. DFP did not produce azotemia.

Cause of death. It is clear that most of the important systems of the body are involved in the effects of poisoning by DFP: The central nervous system, the autonomic nervous system, the respiratory mechanism, the heart, the circulation, the skeletal muscle, the gastro-intestinal tract, the salivary glands, the pilomotor system, and the eye.

The major action which leads to the changes in these systems is, in all probability, the inhibition of the cholinesterase, but there still remains the question as to the weakest link in the chain which leads to death after fatal doses of DFP. It has been assumed that death results from bronchiolar constriction and contractures of the respiratory muscles (7).

In the terminal stages of poisoning with DFP cats present profound depression of respiration, and the blood pressure is at the shock level. The exact site of action which brings this state about is not yet clear. In view of the marked parasympathetic stimulation, the possibility of suffocation due to excessive bronchial secretion was considered. At autopsy, however, examination of the bronchi and lungs failed to reveal any significant amounts of secretion. They failed to show pulmonary edema. Bronchospasm as a cause has not been excluded, although the absence of wheezing makes this appear unlikely. It seems improbable that blocking of neuromuscular transmission is the cause of death since the animal is in a state of collapse before significant impairment of neuromuscular transmission is demonstrable. It is also improbable that the heart is a primary seat of trouble. It seems more likely, in the face of the results up to the present time, that the cause of the deterioration of respiration and circulation lies in an action on the central nervous system. The occurrence of protracted symptoms and instances of damage which appear to be permanent suggest that something more than a primary inhibition of cholinesterase operates in the poisoning by DFP. This factor may be responsible for the fatalities occurring after 10 days or 2 weeks. We have no information concerning the nature of this factor.

The relation between the course of poisoning by DFP and changes in cholinesterase activity is the subject of other papers in the series having to do with the pharmacology of DFP.

SUMMARY AND CONCLUSIONS

1. The effects of di-isopropyl fluorophosphate (DFP) were studied in about 200 cats.

2. DFP produces the following general effects: Motor unrest, muscular twitching, tremors, ataxia, weakness, pilomotor stimulation, respiratory stimulation, salivation, defecation, diarrhea, convulsions, respiratory depression, and death. The symptoms fall ito two groups, "muscarine-like," and "nicotine-like." Death appears to result from a combination of respiratory and circulatory failure.

3. There is a brief latent period of from 1 to 18 minutes after the intravenous injection before symptoms appear, and their severity varies with the dose.

4. In normal, non-protected cats, the duration of symptoms is usually fairly brief, the dose resulting in recovery or death within a few hours or less, usually less than 24 hours.

5. Cats which have received very large doses of DFP, and have been protected by other agents against their acute lethal effects, frequently develop a permanent injury with "nicotine-like" symptoms.

6. The duration of action is only partly due to the persistence of the drug.

Cats which have received doses and are free of symptoms, are sensitized to subsequent doses.

7. Specimens of DFP vary in potency and lose potency fairly rapidly in aqueous solutions. The LD50 of one specimen was about 3.6 mg. per Kg., and of another 1.7 mg. per Kg.

8. DFP produces an increase in the tone, rate, and amplitude of contractions of the gut, effects which are abolished by atropine and epinephrine.

9. Advanced poisoning by DFP is associated with impaired skeletal neuromuscular transmission.

10. DFP in large doses often slows the heart in the normal cat. In such cases atropine may produce some acceleration, although bilateral vagotomy was without effect in one case.

11. DFP exerts no immediate direct action on the blood pressure, but in the stage of advanced poisoning the pressure declines gradually to shock levels.

12. Epinephrine produces the typical effect of cardiac acceleration and rise of blood pressure after DFP.

13. DFP markedly sensitizes the vasodepressor response to acetylcholine.

14. The red blood cell count, white blood cell count, hemoglobin, blood N.P.N., and blood creatinine are not affected by DFP.

15. DFP causes miosis of long duration after conjunctival instillation, and sensitivity to miotic stimuli after intravenous injection.

16. The inactivation of cholinesterase is the major action of DFP, but there are other actions which may or may not be secondary to this, which play a part in its toxic effects. While death from moderate doses seems to be related to the "muscarine-like" actions since it is prevented by atropine, the fatal outcome after massive doses in "protected" animals seems to involve a combination of central and peripheral paralysis of a nature similar to that of nicotine.

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THE ACUTE TOXICITY OF DI-ISOPROPYL FLUOROPHOSPHATE

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The history of the development of the fluorophosphates and detailed studies of pharmacological actions are covered by other papers in this JOURNAL. This report is concerned mainly with acute toxicity of diisopropyl fluorophosphate (DFP).

Data on the toxicity of DFP which Smith and Gates (1) assembled from the classified literature plus some additional classified references are reproduced in table 1. However, these earlier experiments were principally screening tests and as can be seen from this table, in most cases too few animals were used to delimit properly the range of the LD50. Moreover, when larger numbers of animals were used, the majority were given doses producing 0% or 100% mortality. The present study was undertaken to provide more precisely defined values for the acute toxicity of DFP in several species.

EXPERIMENTAL METHODS AND RESULTS. The results of the present investigation are presented below together with brief descriptions of the symptoms observed in normal, unanesthetized mice, rats, rabbits, dogs and cats after oral intravenous, subcutaneous or intramuscular administration of freshly prepared DFP solutions. Tables 2 to 6 give the pertinent experimental details for each series. The period of observation for the deaths recorded was 48 hours. The LD50's were calculated by Bliss' method for small numbers of animals (3).

The following precautions to be observed when working with DFP may be helpful to other laboratories.

1. *Protection against the vapor.* Fluorophosphates are moderately volatile and without much odor at concentrations which may be dangerous after several hours exposure. Since they are slowly detoxified, contact with fluorophosphates should be avoided for several weeks after an exposure which produces even mild symptoms such as pupillary constriction, chest pains, nausea or diarrhea.

2. *Storage of the pure compound.* DFP should be stored in tightly sealed, hard glass containers since deterioration, which may be detected by varying degrees of brownish-pink discoloration of the original, clear, colorless liquid, occurs on continued contact with soft glass.

3. *Solutions for biological studies.* Because the half-hydrolysis time of DFP is about 16 hours in aqueous solutions (2), non-aqueous solvents are preferable. Peanut oil solutions were used for intramuscular injections. Redistilled propylene glycol has been a satisfactory vehicle for oral and intravenous administration. Since propylene glycol is toxic,

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[1] 1st Lt., MAC, AUS.
[2] 1st Lt., SnC, AUS.
[3] 1st Lt., CWS, AUS.

the volumes administered must not be too large. Absorption of water vapor by the propylene glycol must be avoided. Even with these anhydrous solvents, solutions, other than peanut oil, were prepared on the day of the experiment.

Mice. After the subcutaneous administration of a freshly prepared solution of DFP in normal saline to Carworth Farms (Strain CF 1) white mice (18 to 28 grams), tremors and skeletal muscle fibrillations usually were noted within

TABLE 1

The acute toxicity of DFP

SPECIES	ROUTE OF ADMINISTRATION	VEHICLE	NO. OF ANIMALS	ESTIMATED LD50	REFERENCE
				mgm./kgm.	
Monkey	Intravenous	Saline	5	0.25–0.30	12
Rabbit	Intravenous	Saline	27	0.4	5
	Subcutaneous	Water*	8	1.0	6
	Intraocular	Pure	8	1.15–1.4	7
	Intraocular	Triacetin or propylene glycol	8	>1.0	7
	Cutaneous	Pure	2	>117	5
	Oral	Water*	4	4.0	6
Rat	Intramuscular	Water	51	2.0	8
	Subcutaneous	Water*	16	3.0	6
	Oral	Water	10	5–10	9
	Oral	Water*	16	6.0	6
Mouse	Subcutaneous	Water*	26	4.0	6
	Cutaneous	Pure	10	72.0	10
	Oral	Water*	12	2.0	6
Goat	Intravenous	Saline	17	0.8	5
	Subcutaneous	Water*	10	1.0	6
Dog	Subcutaneous	Water*	3	3.0	6

* Presumably water was the vehicle for administration of DFP, although no definite statement could be found in this reference.

15 minutes. With fatal doses, convulsions and respiratory paralysis followed quickly. Most deaths occurred within two hours. All animals surviving two days eventually recovered. Table 2 contains the relevant data and the calculated LD50's for mice for subcutaneous or oral administration of DFP in saline or water solutions respectively.

Rats. Normal Wistar Institute white rats (200–300 grams) were intramuscularly injected with DFP in peanut oil. Within thirty minutes they became weak. After sixty minutes, fibrillary twitchings of the skeletal muscles, increased salivation, retching, diarrhea and micturition occurred. The majority of rats

died from respiratory failure within 2 hours. Rats surviving high doses (>1.5 mgm./kgm.) for more than two hours developed nearly complete paralysis in addition to the above symptoms. Chromodacryorrhea was usually present. Cholinergic symptoms persisted for several hours and then gradually diminished; the paralysis generally persisted and deaths continued to occur during several days. The experimental data and the calculated LD50 are listed in table 3.

Rabbits. Rabbits (1.5–2.5 kgm.) of mixed breeds were intravenously injected with DFP in propylene glycol. Some muscarinic effects were observed but the

TABLE 2

Acute toxicity of DFP after subcutaneous injection or oral administration in mice

ROUTE	DOSE	MORTALITY	LD50
	mgm./kgm.		*mgm./kgm.*
Subcutaneous	2.82	1/15	
	3.55	9/20	3.71 ± 0.16*
	4.47	12/15	
Oral	30.00	1/15	
	35.00	4/15	36.8 ± 0.98*
	40.00	12/15	

* Standard error.

TABLE 3

Acute toxicity of DFP after intramuscular injection in rats

DOSE	MORTALITY	LD50
mgm./kgm.		*mgm./kgm.*
1.26	0/10	
1.33	0/10	
1.58	6/10	1.82 ± 0.09*
2.00	6/10	
2.51	8/10	
3.00	10/10	

* Standard error.

principal signs were observed in skeletal muscle. Within a few minutes after injection, local twitching began in various muscles with the result that incoordinate waves of contraction appeared to pass over the body surface. Ataxia and increased respiration also occurred. In the rabbits more severely affected, flaccid paralysis of the forelimbs and neck ensued within 5 to 30 minutes, accompanied in some animals by some spasticity of the hind limbs. At intervals, there were tonic and clonic convulsions in the most severely affected animals.

Rabbits which became prostrate generally died within 1 or 2 hours after injection, apparently from spastic paralysis of the respiratory muscles and respiratory depression. Some exhibited only flaccid paralysis of the forelimbs and neck and usually died later. Death in these animals followed gradual

failure of the respiration or, less often, starvation due to palsy of the neck muscles. The weakness of the neck muscles produced a characteristic minimal residual sign in rabbits which survived DFP intoxication: such animals tended to rest their heads on some object above the floor level for long periods of time, although able to support their heads voluntarily if necessary.

The experimental data and the calculated LD50's of DFP for oral (aqueous solution) and intravenous (propylene glycol) administration to rabbits are given in table 4.

Dogs. Mongrel dogs (4.5–10 kgm.) were intravenously injected with DFP in propylene glycol. Severe muscarinic and nicotinic effects were observed. Local skeletal muscle contractions were followed by increasingly severe, coarse tremors, increased and labored respiration, and ataxia. There were also profuse

TABLE 4

Acute toxicity of DFP after intravenous and oral administration in rabbits

ROUTE	DOSE	MORTALITY	LD50
	mgm./kgm.		*mgm./kgm.*
Intravenous	0.30	2/10	
	0 35	12/20	0.34 ± 0 01*
	0 4	10/12	
	0 5	10/10	
Oral	5 0	0/10	
	7.5	3/10	
	10 0	7/14	9.78 ± 0.65*
	12 5	11/15	
	15 0	10/11	

* Standard error.

salivation, vomiting, wheezing respiration and diarrhea. In some dogs the tremors became sufficiently intense to prevent locomotion. Finally, there was a phase of hyperextension with prostration accompanied by convulsions. The animals which became prostrate within 35 minutes after injection generally died despite lessening of the excitatory effects if the animal survived several hours. The terminal signs of delayed deaths were not observed. The animals which recovered, recovered gradually, generally within 4 days. The pertinent data and the calculated LD50 are presented in table 5.

Cats.[4] After the intravenous injection of DFP solutions in propylene glycol in cats, symptoms appeared within 1 to 18 minutes The acute symptoms included motor unrest, muscular twitching, ataxia, weakness, pilomotor stimulation, panting and labored respiration, salivation and defecation. Dyspnea and unrest increased in severity and death was sometimes preceded by severe con-

[4] These data were obtained by Dr. McKeen Cattell and associates in the Department of Pharmacology, Cornell University Medical College Their full report occurs elsewhere in this JOURNAL (4).

vulsions. When death was delayed, the outstanding symptoms were prostration and shallow or labored respiration.

There was marked individual variation with respect to duration of symptoms and survival time. In general survival time was shorter as the dose was increased. Animals receiving smaller fatal doses sometimes survived for several hours, during which time most of the acute symptoms subsided and weakness, ataxia and pilomotor stimulation predominated. The same symptoms occasionally persisted for several days in animals which eventually recovered. The

TABLE 5

Acute toxicity of DFP after intravenous injection in dogs

DOSE	MORTALITY	LD50
mgm./kgm.		*mgm./kgm.*
1.80	0/7	
2.40	2/7	
2.77	1/10	3.43 ± 0.62*
3.20	5/10	
3.79	3/4	

* Standard error.

TABLE 6

Acute toxicity of DFP after intravenous injection in cats

DOSE	MORTALITY	SURVIVAL TIME	LD50
mgm./kgm.		*minutes*	*mgm./kgm.*
1.0	0/12		
1.5	9/24	27–270	
1.75	6/6	17–131	
2.0	6/10	21–170	1.63 ± 0.03*
3.0	13/13	13–38	
5.0	10/10	4–7	
7.0	6/6	4–5	
10.0	2/2	3–4	

* Standard error.

findings are summarized in table 6. The calculated LD50 for DFP intravenously administered in these cats is 1.63 mgm./kgm. with a standard error of ±0.03 mgm./kgm.

Discussion. Since DFP is an anticholinesterase, its effects are cholinergic. Nicotinic effects were pronounced in all species with variations as to degree and type. Marked muscarinic effects occurred in all species except the rabbit.

In general, for a particular vehicle, route of administration and species, the more rapidly toxic signs developed, the more severe were the eventual effects and the greater was the probability of death. The median time of death decreased with increase in dose. At doses below those producing 100% mortality, the majority of deaths occurred within the first 2 hours, presumably due to bron-

hiolar constriction and contractures of the respiratory muscles (11). In general animals which became prostrate died. Few deaths occurred after 48 hours.

Toxicity data on DFP previously reported in classified literature were primarily from screening tests and not sufficiently precise to delimit accurately the LD50 and its range. The results of the present study are more extensive and the LD50's and standard errors were calculated by the method of Bliss. These data agree fairly well with those previously reported (cf. table 7). except for the oral LD50's for rabbits and mice. The large standard error for the intravenous LD50 for dogs may be due to the heterogeneity of the dogs as regards breed, size and age.

TABLE 7

*Comparison of LD50 (mgm./kgm.) for various species and routes of administration**

SPECIES	ROUTE OF ADMINISTRATION					
	Intravenous	Subcutaneous	Intramuscular	Cutaneous	Intraocular	Oral
Monkey	0 25–0.30 (12)					
Rabbit	0 4 (5) 0.34 (*)	1.0 (6)		>117 0 (5)	1.2 (7)	4.0 (6) 9.78 (*)
Rat		3 0 (6)	2.0 (8) 1 82 (*)			6.0 (6) 5–10 (9)
Mouse		4.0 (6) 3.71 (*)		72.0 (10)		2.0 (6) 36.8 (*)
Goat	0.8 (5)	1 0 (6)				
Dog	3 43 (*)	3 0 (6)				
Cat	1 63 (*)					

* References so marked are data from the present study. Others in parentheses are cited in the bibliography

The great variation in susceptibility of different species to DFP is especially notable. The intravenous LD50 for the dog was about twice that for the cat and about ten times that for the rabbit. This is in contrast with a rather narrow range of individual susceptibilities to DFP within a species. The latter is exemplified by the small coefficients of variation which reflect the steep slopes of the dosage-mortality curves for the different series of the present study.

SUMMARY

1. The cholinergic signs observed in normal unanesthetized mice, rats, rabbits, dogs and cats after administration of di-isopropyl fluorophosphate (DFP) by different routes of administration have been described briefly.

2. The acute median lethal doses of DFP after intravenous administration in

52 rabbits, 38 dogs and 83 cats; subcutaneous injection in 50 mice, intramuscular injection in 60 rats; and oral administration in 45 mice and 50 rabbits have been determined.

3. The LD50's and the standard errors for the several series of experiments were as follows:

a. Mice	Oral	36.8 mgm./kgm. ± 0.98
	Subcutaneous	3.71 mgm./kgm. ± 0.16
b. Rats	Intramuscular	1.82 mgm./kgm. ± 0.09
c. Rabbits	Oral	9.78 mgm./kgm. ± 0.65
	Intravenous	0.34 mgm./kgm. ± 0.01
d. Dogs	Intravenous	3.43 mgm./kgm. ± 0.62
e. Cats	Intravenous	1.63 mgm./kgm. ± 0.03

The authors wish to thank Miss Eleanor B. Donohue for her assistance in statistical analysis of the data.

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THE RELATIONSHIP BETWEEN CHOLINESTERASE INHIBITION AND THE PHARMACOLOGICAL ACTION OF DI-ISOPROPYL FLUOROPHOSPHATE (DFP)

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A large number of chemically unrelated drugs is known to inhibit cholinesterase. To this property have been attributed some or all of their pharmacological actions. The more potent anticholinesterase agents include eserine, prostigmine and related basically substituted phenylesters of monoalkyl carbamic acids (1, 2), methylene blue (3), atabrine (4) and the fluoride ion. In addition, several alkaloids possess this action to various degrees (5). The alkyl fluorophosphate esters, originally synthesized by Lange and Von Krueger in 1932 (6), are the first group of compounds known to inactivate cholinesterase irreversibly.

The similarity between the actions of the alkyl fluorophosphates and eserine was first noted by McCombie and coworkers (7), and the irreversible anticholinesterase activity of the former was demonstrated by Mackworth (8). Mazur and Bodansky (9) have conducted an intensive investigation of the mechanism of this enzyme inactivation. The general pharmacology of di-isopropyl fluorophosphate (DFP), the most potent member of the group, has been studied by Cattell and coworkers (10) and Bowers (11).

The irreversible nature of the inactivation produced by DFP permits for the first time a simple accurate estimation of the true degree of inhibition of blood and tissue cholinesterase activity following the administration of an anticholinesterase drug. Since the return of cholinesterase activity can then result only from the formation of new enzyme, its rate of synthesis in different tissues can also be followed. The study of symptomatology at different levels of cholinesterase activity in the blood and tissues should clarify the present indefinite knowledge of this relationship.

In the present study, these factors have been investigated to provide a pharmacological background for the clinical testing of the efficacy of DFP in the treatment of myasthenia gravis. The possible superiority of DFP over other anticholinesterase drugs in treating this disease is suggested by its prolonged effect and its observed production of a predominance of nicotinic over muscarinic actions in experimental animals. The relationship between symptomatology and the degree of inhibition of cholinesterase in the blood and tissues has been studied in order to determine the value of following blood cholinesterase levels as a dosage guide. Also, the suitability of peanut oil as a vehicle for the administration of DFP and certain factors affecting the absorption of the drug have been examined.

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I. IN VITRO INHIBITION OF CHOLINESTERASE OF RAT AND DOG TISSUES BY DI-ISOPROPYL FLUOROPHOSPHATE. It was found by Mazur and Bodansky (9) in their work on the rabbit, monkey and human that for a given species the cholinesterases of the red cells and brain are nearly equally sensitive to di-isopropyl fluorophosphate, while serum cholinesterase is markedly divergent. This is in agreement with the recent report of Nachmansohn and Rothenberg (12) in which nervous tissue and erythrocytes were shown to contain "specific cholinesterase," while the acetylcholine-splitting enzyme of the serum was found to be an unspecific esterase. Such being the case, measurements of the degree of inhibition of red cell cholinesterase following the administration of DFP in vivo should provide an indication of the activity of the enzyme in the brain, assuming the uptake of DFP to be approximately equal in both types of cells. Inasmuch as dogs and rats were also to be used in these studies, in vitro measurements of the sensitivity of cholinesterase to DFP were made on dog serum, red cells and brain, and rat serum, red cells, brain and muscle.

METHODS. Rat brain homogenates were prepared by rapidly removing whole brains from the decapitated animals, weighing, and grinding in the Potter-Elvejhem homogenizer in the presence of a magnesium chloride-sodium bicarbonate solution (4 parts 0.04 M. $MgCl_2$ + 1 part 0.15 M $NaHCO_3$) and a small amount of powdered silica. Two rinsings were added plus sufficient solution to make a 1:10 suspension. The muscle homogenate was prepared similarly from the gastrocnemius, which was first freed of nerve insofar as possible and chopped with scissors before grinding. Because of its far lower enzyme activity the final concentration was made 1:5. The whole dog brain was ground in the Waring blender with four times its volume of 0.025M. sodium bicarbonate. The blood of both dogs and rats was defibrinated, the serum removed, and the red cells washed twice with saline before being hemolyzed by the addition of two times their volume of distilled water. Cholinesterase activity was determined in the Warburg apparatus using slight modifications of the method of Ammon as described by Mazur and Bodansky (9). CO_2 production was followed for ten to sixty minutes (a selected time interval was used throughout the determinations for a given tissue), and cholinesterase activities were calculated as percentages of the values obtained from the control vessels containing no DFP. A blank containing only $NaHCO_3$ and acetylcholine bromide was run each time to correct for non-enzymatic hydrolysis, and it was found necessary to run an additional control containing no acetylcholine bromide with each hemolysate to account for acid production which was invariably observed in these preparations.

RESULTS. Figure 1 indicates that in the rat, the brain and muscle cholinesterases are considerably more sensitive to DFP than is the enzyme of the red cells. In the dog (fig. 2) the reverse is true, the cholinesterase of the brain being more resistant to inhibition by DFP than is the erythrocyte cholinesterase. In both species the serum cholinesterase shows inactivation at far lower concentrations of DFP than are required to inhibit the enzyme of brain, muscle or erythrocytes. The flattening of the lower portions of the curves before approaching the base-line, which is particularly apparent with the rat erythrocytes and dog serum, is attributed to two factors: (1) the presence of a non-specific esterase which is not affected by DFP, and (2) the hydrolysis of DFP itself by the "phosphofluorase" activity of the serum (13).

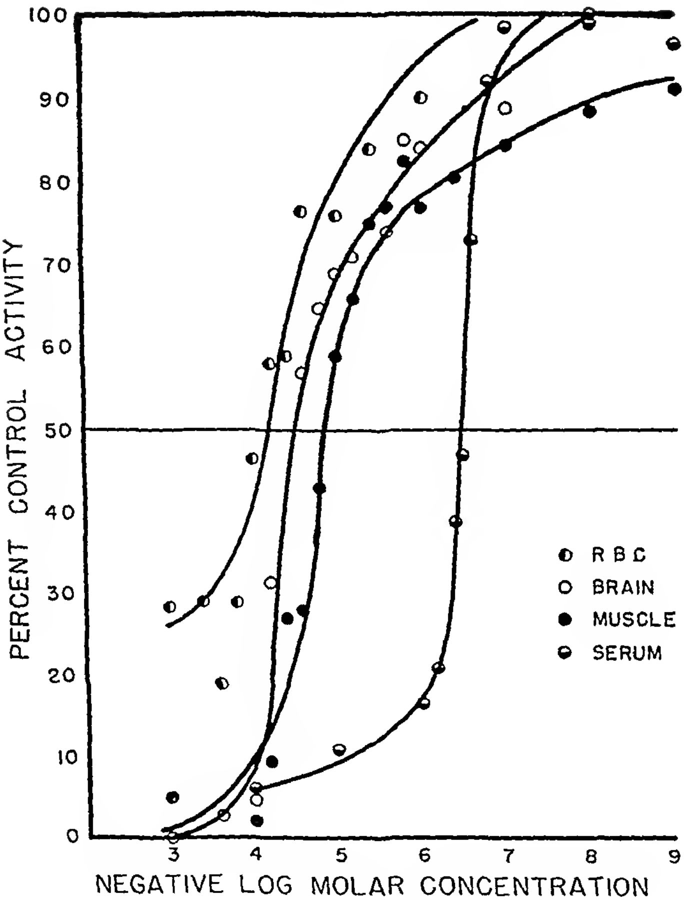

FIG 1 THE INHIBITION OF RAT CHOLINESTERASE BY DI-ISOPROPYL FLUOROPHOSHATE IN VITRO

The "pK values" (negative logarithm of the molar concentration of DFP required to produce 50% inactivation) were found to be as follows:

	Rat	*Dog*
Serum	6 5	6 8
Red cells	4 2	4 3
Brain	4 5	3 8
Muscle	4 8	

II. IN VIVO INHIBITION AND REGENERATION OF CHOLINSTERASE AS RELATED TO SYMPTOMATOLOGY IN RATS, DOGS, AND MONKEYS FOLLOWING THE INJECTION OF DI-ISOPROPYL FLUOROPHOSPHATE. It has previously been shown that very low

values for serum cholinesterase can be produced by DFP in man and experimental animals with practically no associated symptomatology, but following amounts sufficient to inhibit brain and red cell cholinsterase markedly, muscarinic and nicotinic symptoms appear (9). The regeneration of serum cholinesterase is

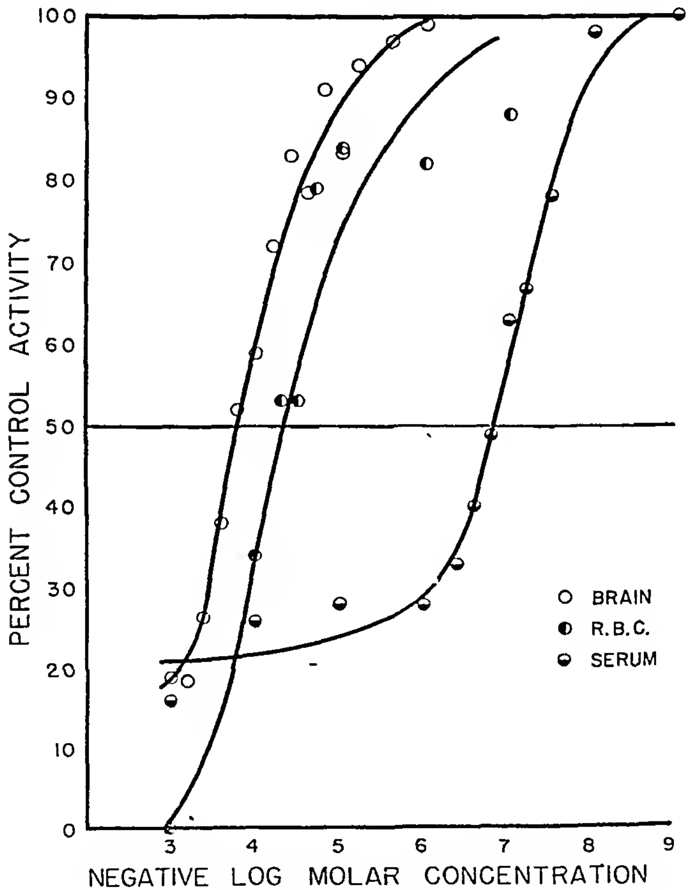

FIG. 2. THE INHIBITION OF DOG CHOLINESTERASE BY DI-ISOPROPYL FLUOROPHOSPHATE IN VITRO

relatively rapid, whereas the red cell cholinesterase has been found to remain at a low value for several weeks. Inasmuch as DFP inrreversibly inactivates cholinesterase, the return of cholinesterase activity must be attributed to a resynthesis of the enzyme. For these reasons it was of interest to determine simultaneously the rates of resynthesis of cholinesterase in a variety of tissues. The rat was employed for such a study, the relative rates of regeneration of cholinesterase being followed in serum, red cells, brain and muscle

Regenration of tissue cholinesterase in rats. One hundred female rats, each weighing approximately 200 grams, were injected with 1.0 mgm. DFP/Kgm. (ca. one-half the LD50). The drug was dissolved in peanut oil (0.1% solution) and injected into the left gastrocnemius. The rats were sacrificed in groups of six for determinations of the cholinesterase activities of the serum, red cells, brain and right gastrocnemius at intervals of 5 hours and 1, 3, 5, 10, 15, 20, 31, 40, 49, 60, 70, 85, and 98 days. Measurements run on the brains and gastrocnemii of six uninjected animals fell within fairly close limits and the average

TABLE 1

Cholinesterase regeneration in rats following the administration of di-isopropyl fluorophosphate

All animals received 1 0 mgm. DFP/kgm. intramuscularly. Each figure represents the average value for six rats.

TIME SACRIFICED AFTER INJECTION	PER CENT CONTROL CHOLINESTERASE ACTIVITY*			
	Serum	Erythrocytes	Muscle	Brain
5 hours	4 ± 3.7†	43 ± 13.9	18 ± 4.3	9 ± 5.9
1 day	11 ± 3.1	44 ± 9.3	17 ± 8.9	11 ± 8.2
3 days	50 ± 8.7	54 ± 7.4	40 ± 10.0	26 ± 2.6
5 days	62 ± 13.9	62 ± 15.3	41 ± 9.4	37 ± 2.8
10 days	72 ± 18.1	78 ± 5.0	50 ± 10.8	46 ± 3.2
15 days	93 ± 17.1	112 ± 3.6	53 ± 7.1	54 ± 3.0
20 days	115 ± 16.4	107 ± 19.6	69 ± 2.3	62 ± 1.6
31 days	104 ± 23.0	109 ± 15.7	66 ± 8.2	71 ± 3.3
40 days	79 ± 32.0	96 ± 13.5	71 ± 6.3	79 ± 7.8
49 days	115 ± 25.6	103 ± 10.7	76 ± 12.1	79 ± 5.6
60 days	143 ± 48.3	141 ± 29.7	83 ± 10.3	85 ± 3.0
70 days	117 ± 43.2	103 ± 8.6	80 ± 10.9	82 ± 3.6
85 days	94 ± 17.1	103 ± 16.0	96 ± 14.4	88 ± 5.3
98 days	115 ± 56.1	95 ± 13.4	92 ± 13.5	97 ± 6.1

* Control brain and muscle cholinesterase activites (in cu. mm. CO_2 liberated/100 mgm. wet weight tissue/30 minutes): Brain: 442 ± 57; Muscle: 34.0 ± 4.3.

† Standard deviation: $\sqrt{\frac{\Sigma d^2}{(n-1)}}$.

values served as controls. The necessity for using the entire brain and muscle in such a study arises from the fact that cholinesterase distribution is not uniform in these tissues; in the brain there is a great variation between different lobes(14) and in the muscle the concentration is greatest at the sites of the myoneural junctions (15). Since the animals exhibited marked individual variation in respect to serum and red cell cholinesterase activity, it was necessary to do a control determination on each rat, using a blood sample taken before the injection of DFP. The same procedures were used for preparing and assaying the tissues as referred to above.

The dose employed was fatal to 6 of 100 rats. Symptoms were maximal approximately 5 hours after injection and consisted of fasciculation of the skeletal

and lingual muscles, generalized depression and occasionally chromodacryorrhea. All animals were symptom free within 24 hours.

Five hours after the injection of DFP the cholinesterase activities of the serum, red cells, muscle and brain were 4, 43, 18 and 9 per cent of normal respectively (table 1). With the exception of the unusually low figure for brain, these values are in line with the relative sensitivities to DFP found in vitro. Due to the high lipoid content of the brain, it might be expected to take up a relatively greater amount of a lipoid-soluble agent. The serum and red cell cholinesterases were rapidly regenerated, returning to control values in approximately two weeks. In brain and muscle, the initial regeneration rates were high, so that within three days the cholinesterase activities were more than twice what they had been following the initial inhibition. After this the return was much slower, and approximately one hundred days were required for the complete restoration of the normal enzyme activity.

In vivo inhibition and regeneration of cholinesterase in the dog and monkey. It can be seen from the above data that severe symptoms associated with the inhibition of cholinesterase are apparent only when tissue cholinesterase values reach a low level. Moreover animals became symptom free when only a fraction of the normal amount of enzyme was present. Whereas the rat affords a convenient species for the study of tissue cholinesterase, symptomatology can be more advantageously followed in the dog and monkey. It is not feasible in these animals to follow the progressive regeneration of brain and muscle cholinesterase. However the enzymatic activity of serum and red cells can be studied in respect to dosage and symptoms. The same applies to humans. Thus in this manner dosage in humans might conceivably be controlled by objective measurements of cholinesterase activity. The monkey is particularly suited for such a study inasmuch as Mazur & Bodansky (9) have shown that in this species the in vitro sensitivities of brain, red cells, and serum cholinesterases to DFP are practically identical to esterases from the same sources in humans.

PROCEDURE. Nine dogs and four monkeys (Macaca mulatta) were given single intramuscular injections of DFP in peanut oil in doses ranging from 0.05 to 5.0 mgm./kgm. and 0.05 to 0.3 mgm./kgm. respectively. Control values for serum and red cell cholinesterase activity obtained beforehand showed great individual variation between different animals but consistent values from day to day in a given animal. Signs of intoxication and the degree of inhibition and rate of regeneration of serum and red cell cholinesterase were followed. The first sample in the dogs was taken 24 hours after injection and observations were continued until recovery was complete. The monkeys were followed at more frequent intervals.

Dogs. In agreement with the in vitro inhibition curves, it was found that at the lower dosage range (0.05–1.0 mgm. DFP/kgm.) the inhibition of serum cholinesterase 24 hours after injection was greater than that of the red cell cholinesterase (table 2). At higher doses (1.0–5.0 mgm./kgm.) the erythrocyte cholinesterase showed further inhibition, exhibiting zero activity at the highest dose employed, while the serum retained a substantial portion of its activity due to its rapid regenration rate and the presence of the non-specific enzyme fraction. The red

cell cholinesterase returned slowly. In dog D (5.0 mgm./kgm.) it was regenerated to approximately 90 per cent of normal after one hundred days. Only four animals showed symptoms: dogs K (2.0 mgm./kgm.), B (3 0 mgm./kgm.), C (4.0 mgm./kgm.) and D (5.0 mgm./kgm.). Dog K, the red cell cholinesterase activity of which fell to 14% the first day after injection, gave evidence of slight nicotinic action consisting of uncoordinated skeletal muscle fasciculation, which disappeared completely after the fifth day. The animal's normal activities were in no way impaired and no muscarinic effects were observed. During this period

TABLE 2

Inactivation and regeneration of serum and erythrocyte cholinesterase in dogs following single intramuscular injections of DFP in oil

DOG	DOSE	ENZYME SOURCE	PER CENT ORIGINAL CHOLINESTERASE ACTIVITY ON DAYS INDICATED FOLLOWING ADMINISTRATION OF DFP																				
			1	2	3	5	7	8	9	10	12	13	14	17	19	23	24	31	36	38	46	58	80
	mgm / kgm																						
S	0 05	Serum	42	56	64	82	84		75		78		83	83	98								
		RBC.	101	94	103	99	101		114		101		101	95	110								
T	0 10	Serum	36	50	60	83	105		97		100		107	112	99		102						
		RBC	84	83	95	85	80		91		103		103	112	112		94						
E	0 30	Serum	41	61	67	84	95	98		109	103		112					108					
		RBC	84	86	86	86	83	87		95	101		92					91					
U	0 50	Serum	39	53	60	75	82		80		83		88	87	84		98						
		RBC	75	70	81	79	75		84		84		79	99	94		86						
G	1 00	Serum	28	59	69	84	90		102				104	84	98			106		102	108		
		RBC.	50	46	45	38	42		54				62	55	59			72		88	100		
K	2 00	Serum	43	52	69	57	89			87		101			104	104				102		93	
		RBC	14	12	9	20	15			36		41			58	66				88		84	
D	5 00	Serum	11	50	64			87		101		95				90		96	102			99	100
		RBC.	0	0	5			5		2		19				32		39	45			68	92

there was a slight rise in red cell cholinesterase activity and the serum cholinesterase activity rose from 43 to approximately 80 per cent. Dogs B and C succumbed to the effects of the drug before the 24 hour sample was obtained Dog D exhibited an unusually high resistance to the toxic effects of DFP, as it recovered from a dose of 5.0 mgm./kgm. The day following the injection its serum cholinesterase activity had fallen to 11% and the red cell activity to zero. This dog presented severe nicotinic symptoms. When the animal was lying down in complete relaxation there was little indication of abnormality, but on standing or attempting to move extreme muscular tremors and fasciculation ensued, along with a persistent priapism. Forcing the dog to walk resulted in spastic

paralysis, from which it relaxed slowly. After a few days it became conditioned to this state and preferred to remain as motionless as possible. Its appetite remained normal in spite of the initial difficulty it had in eating, and copious amounts of water were consumed. Most unexpected was the nearly complete absence of any muscarinic symptoms (e.g. miosis, bradycardia, diarrhea) and only a slightly excessive degree of salivation was observed. This was increased noticeably at one time when the dog struggled during blood sampling and was presumably due to an excessive quantity of acetylcholine being poured into the circulation as a result of increased activity. The nicotinic symptoms gradually decreased in severity and by the tenth day were scarcely noticeable, at which time the serum cholinesterase had returned to normal but the erythrocyte cholinesterase was only 2 per cent of the control value.

Monkeys. Monkeys A and B (0.05 and 0.1 mgm. DFP/kgm. intramuscularly) showed no symptoms. Monkey C (0.20 mgm./kgm. i.m.) developed marked muscular fasciculation, particularly in the lingual, trapezoid and deltoid muscles, about one hour after injection, at which time the serum cholinesterase activity had fallen to zero and the red cell cholinesterase to 11% of its original activity. Fasciculation gradually spread to the muscles of the face, legs, and abdomen and eventually became generalized. Diarrhea was present but there was no miosis, excessive salivation, bradycardia, or apparent bronchial constriction; respiration was rapid and shallow. When the animal was returned to its cage, it was extremely exhausted and its attempts to climb were poorly coordinated. By the fourth day the nicotinic symptoms were gone but the monkey had become extremely dehydrated, emaciated and weak. In spite of repeated saline and glucose infusions death ensued on the fifth day.

Monkey D (0.30 mgm./kgm. i.m.) showed an early course similar to that of Monkey C. Thirty-nine minutes after injection, when the serum cholinesterase activity had fallen to zero and the erythrocyte to 3%, nicotinic fasciculation first appeared and followed the same pattern as in the previous animal. One hour later salivary flow became copious and the presence of distinct rales and wheezing indicated bronchial constriction; respiration was dyspneic. The animal died in respiratory failure 108 minutes after injection, and autopsy showed the air passages to be filled with frothy fluid. Cholinesterase determinations run on a blood sample collected immediately after death gave zero values for both serum and red cells.

The cholinesterase of the serum and red cells was regenerated at a much slower rate in this species than in the rats or dogs (table 3).

Discussion. The above findings emphasize the fact that the overt behavior of an organism may be completely normal when the cholinesterase of both the blood and the tissues is inactivated to a very great extent. Judging from the findings in rats, the critical amount necessary in the brain and muscles for animals to remain apparently normal is between 10 and 20 per cent of that initially present. Following inactivation to below this level by DFP, the initial regeneration rates and concomitant disappearance of symptoms were rapid. The regeneration rates then fell off so that several weeks elapsed before the original amounts of cholinesterase were restored.

The regeneration rates of red cell cholinesterase in the dog, monkey and rat deserve special comment. The mammalian erythrocyte, a non-nucleated cell, might be presumed to have little anabolic activity. Following the irreversible inactivation of an enzyme contained in these cells, the reappearance of enzymatic activity can only be expected to be associated with the appearance of newly formed cells in the circulation. Thus the rates of regeneration of red cell cholinesterase should parallel the rates of erythropoiesis. Moreover the time necessary for the return to normal enzymatic activity should be a measure of the life

TABLE 3

Inactivation and regeneration of serum and erythrocyte cholinesterase in monkeys following single doses of DFP in oil

MONKEY	DOSE	ROUTE	ENZYME SOURCE	PER CENT ORIGINAL CHOLINESTERASE ACTIVITY ON DAYS INDICATED FOLLOWING ADMINISTRATION OF DFP																			
				1	2	3	4	5	6	8	16	21	29	33	42	43	54	56	70	86	91	95	119
	mgm / kgm																						
Am	0.05	I M	Serum	9	9		38		52		64		104	88		120	122		117			113	124
			RBC	48	47		54		54		55		57	64		66	71		77			82	97
Bm	0.10	I M	Serum	12	29		24		55		69		67	71		86	91		79			103	96
			RBC	21	18		25		29		54		67	76		87	91		109			111	103
Cm	0.20	I M.	Serum	4		58		35															
			RBC	0		4		4															
Dm	0.30	I M	Serum	0																			
			RBC	0																			
Em	0.10	Oral	Serum	44	53		77		91	97		109	104		111						113		
			RBC	99	107		103		102	109		103	101		104						96		
Fm	0.20	Oral	Serum	5	35		66		89	97		137	105		100						105		
			RBC	54	52		48		55	66		79	82		87						97		
Gm	0.30	Oral	Serum	8	29						68	62											
			RBC	22	23						54	61											
Hm	0.50	Oral	Serum	7	60						95	87	91					96		97			
			RBC	21	24						45	53	58					71		85			

duration of the erythrocyte Figures that have been obtained for the life durations of the erythrocytes in the monkey, dog, and rat are 120 days (16), 97–117 days (17) and 8–9 days (18) respectively. These figures agree closely with the regeneration times of erythrocyte cholinesterase activity presented in tables 1, 2 and 3 The rapid regeneration of inactivated serum cholinesterase is comparable to the rate of formation of serum proteins in general (19). The normal function of the cholinesterase of the serum and red cells remains a matter for conjecture.

The relative absence of muscarinic actions after doses of DFP which produce extreme nicotinic effects may be the result of enhanced sympathetic activity. It may be assumed that DFP inactivates the cholinesterase of all autonomic ganglia, including the adrenal medualla and those of the sympathetic nerves. In

the monkey which received the highest dose of DFP, muscarinic actions, including diarrhea, excessive salivation and bronchoconstriction, did become marked.

The limitations of the use of the degree of inhibition of serum and red cell cholinesterase activity as an index of the enzymatic activity in the tissues are clearly evident from the above data. The great susceptibility of serum cholinesterase to inhibition by DFP, especially in the monkey, makes possible the nearly complete inactivation of this enzyme at dose levels which have negligible effects on tissue cholinesterases as judged by the cholinesterase activity of erythrocytes and absence of symptoms. Thus the futility of relating the pharmacological actions of an anticholinesterase drug to its effects on serum cholinesterase is apparent. Likewise the clinical significance of variations in serum cholinesterase may be questioned. On the other hand in vitro susceptibility of the cholinesterase of erythrocytes is of the same order of magnitude as that of brain and muscle. As a result there is a relationship between the degree of inhibition of erythrocyte cholinesterase activity and symptomatology. However the difference in the rates of regeneration of the cholinesterase in red cells and other tissues limits the use of erythrocyte cholinesterase as an index. For example, in dog D there was complete remission of symptoms ten days after the administration of DFP. During this interval no appreciable return of erythrocyte cholinesterase activity was detected. The slow regeneration of red cell cholinesterase also permits this enzyme to be inactivated by repeated small doses of DFP which have little cumulative effect on the cholinesterase of tissues and serum which are more rapidly replaced (2)).

III. Studies on the stability and absorption of solutions of di-isopropyl fluorophosphate in oil. DFP has been found to hydrolyze rapidly in buffered and unbuffered aqueous solutions with the production of hydrofluoric and di-isopropyl phosphoric acids (21). The instability of aqueous solutions prompted the search for a vehicle, suitable for clinical use, in which DFP did not decompose. The fact that DFP, an oily liquid, is completely miscible in oil suggested the use of peanut oil.

The stability of DFP in peanut oil. A peanut oil solution containing 1.0 mgm. DFP per cc. was prepared 25 August 1944 and stored in a rubber-stoppered amber glass bottle at room temperature. Mouse toxicity tests were run on this material at intervals of 0, 8, 28, 40, 52, 105, 287 and 347 days and the results compared with the toxicity of a freshly-prepared control solution. To determine its stability against autoclaving, a solution of the same concentration was prepared in autoclaved peanut oil, using aseptic technique insofar as possible, and sealed in 2.0 cc. ampoules. The following day it was autoclaved for 1 hour at 117°C. and a mouse toxicity test was run against a similarly prepared unautoclaved sample.

Results are presented in table 4. No deterioration was detected after autoclaving for one hour or storage for nearly a year, and the autoclaved solution was found to be sterile with respect to both aerobic and anaerobic organisms. This preparation has been used successfully for intramuscular injections in humans (22).

The rate of intramuscular absorption of DFP in peanut oil. The property of DFP to inactivate cholinesterase irreversibly provided a criterion for following the absorption of the drug when the mode of administration was varied. To determine the influence of concentration on the rate of absorption of DFP from an intramuscular site, two dogs were injected with the same dose (1.0 mgm. DFP/kgm. i.m.) at different concentrations, 5.0 mgm. and 25.0 mgm. per cc.

TABLE 4

The stability of DFP in peanut oil

All solutions prepared as 1.0 mgm. DFP/cc.; assayed by subcutaneous injection in mice.

SOLUTION	TREATMENT	DOSE	NUMBER DEAD IN 24 HRS.
		mgm. DFP/kgm.	
Standard	None	5.0	2/10
		7.5	5/10
		10.0	8/10
A	Autoclaved	5.0	1/10
		7.5	9/10
		10.0	9/10
B	Stored 347 days	5 0	7/10
	Stored 347 days	7 5	5/10
	Stored 347 days	10.0	8/10

TABLE 5

Rate of inactivation of serum and erythrocyte cholinesterase in dogs following intramuscular injection of DFP in oil

DOGS	WEIGHT	DOSE	CONCENTRATION, SOLN INJECTED	ENZYME SOURCE	PER CENT ORIGINAL CHOLINESTERASE ACTIVITY							
					30 min	60 min	120 min	180 min	240 min	8 hrs	24 hrs	4 days
	kgm	*mgm / kgm*	*mgm /cc*									
L	14.4	1 0	5 0	Serum	8	4	4	3	3	8	29	79
				RBC.	48	38	36	23	18	17	20	20
M	15.2	1 0	25.0	Serum	7	6	3		2	12	37	74
				RBC.	35	25	26		25	21	23	21

Blood samples were taken at frequent intervals for the determination of the serum and red cell cholinesterase activities. These values are presented in table 5. The slight differences between the two sets of figures indicate that absorption of the drug took place nearly as rapidly with the dilute as with the concentrated solution. Both dogs showed skeletal muscle fibrillation and excessive salivation approximately one hour after injection and were symptom-free the following day. It is interesting to note that restoration of serum cholinesterase began between four and eight hours after injection.

Estimation of the intramuscular:oral dosage ratio. The ratio between the effective intramuscular and oral doses of DFP was determined by giving a solution of the drug in peanut oil to four monkeys by stomach tube in doses ranging from 0.10 to 0.50 mgm. per kgm. and comparing the resulting cholinesterase levels of

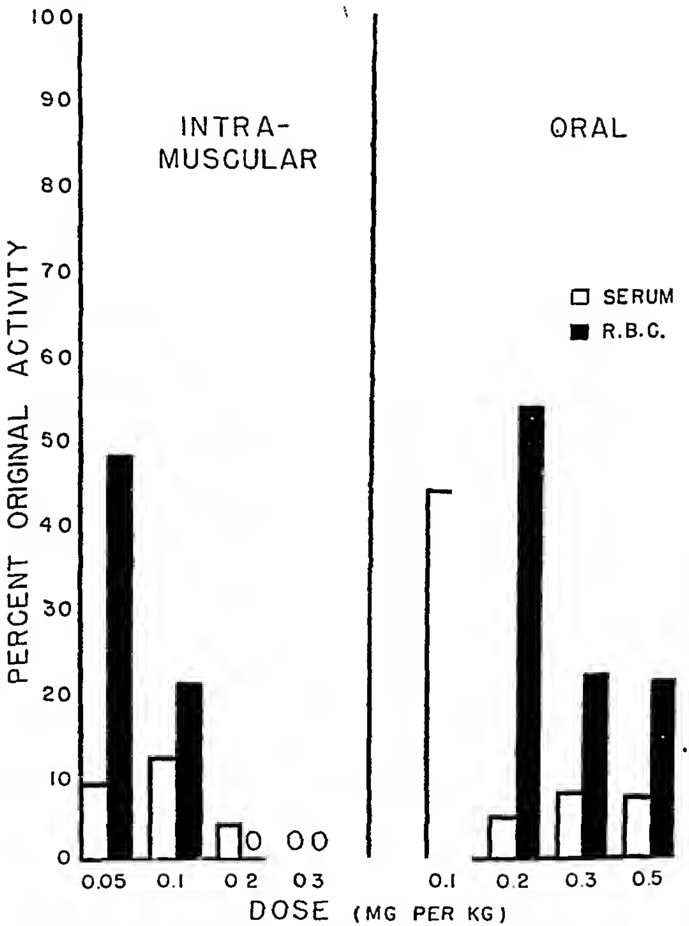

FIG. 3. THE INITIAL INHIBITION OF SERUM AND RED CELL CHOLINESTERASE IN MONKEYS FOLLOWING THE ADMINISTRATION OF DI-ISOPROPYL FLUOROPHOSPHATE

the serum and red cells with those previously found in monkeys receiving intramuscular injections. Hourly blood samples taken in the period immediately following administration showed that on a basis of serum and red cell cholinesterase inhibition, absorption was complete within three hours. Subsequent values are shown in table 3. Monkey H (0.50 mgm. DFP/kgm.) was the only animal in this group in which any sign of DFP action was manifested. This

consisted solely of fasciculation of the lingual muscles, which became apparent two hours after administration and had disappeared by the following day.

A comparison between the initial red cell cholinesterase inhibitions produced by the oral and intramuscular administrations of DFP (fig. 3) indicates an intramuscular:oral dosage ratio of 1:4. This value was confirmed in humans by the following experiment. Three volunteers were given intramuscular injections of 0.5 mgm. DFP/60 kgm. of a sterile peanut oil solution and three others received orally four times this dose in gelatine capsules. Cholinesterase determinations performed on blood samples taken 24 hours after administration gave average serum cholinesterase activities of 39 and 36 per cent of the control

TABLE 6

Inhibition of serum and red cell cholinesterase in humans 24 hours after single doses of di-isopropyl fluorophosphate in peanut oil

SUBJECT	WEIGHT	DOSE	ROUTE	PER CENT ORIGINAL CHOLINESTERASE ACTIVITY	
				Serum	Red blood cells
	kgm	*mgm /60 kgm*			
B M.	95 0	0 5	Intramuscular	42	104
M C	87.5	0 5	Intramuscular	35	102
F P.	85 5	0 5	Intramuscular	40	110
Average.				39	105
R. B	82 3	2.0	Oral	57	96
G. K.	76.3	2 0	Oral	26	108
A. G.	60 8	2 0	Oral	25	107
Average.				36	104

values for the two groups respectively (table 6). The cholinesterase of the erythrocytes was not affected by these doses and no symptoms were noted.

SUMMARY

1. Rats, dogs and monkeys appeared to show no physiological abnormality when the cholinesterase of the blood and tissues was reduced to a low percentage of its normal activity by di-isopropyl fluorophosphate The signs associated with further reduction are described.

2. It was found that determinations of serum and red cell cholinesterase inhibition following the administration of di-isopropyl fluorophosphate do not provide accurate estimates of the activity of the enzyme in the tissues, excepting possibly during the period immediately following a single dose. Such measurements can be used advantageously to follow rates of absorption.

3 No deterioration was detected in solutions of di-isopropyl fluorophosphate in peanut oil following autoclaving for one hour or storage at room temperature for one year.

4. The concentration of a peanut oil solution of di-isopropyl fluorophosphate had little effect on its rate of absorption from an intramuscular site in dogs.

5. The effective intramuscular: oral dosage ratio for di-isopropyl fluorophosphate in peanut oil was found to be approximately 1:4 in monkeys and humans.

We wish to express our appreciation to Lt. Carolyn W. Hammond for conducting the sterility tests.

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THE CHRONIC TOXICITY OF DI-ISOPROPYL FLUOROPHOSPHATE (DFP) IN DOGS, MONKEYS AND RATS

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An investigation of certain of the actions of di-isopropyl fluorophosphate (DFP), conducted as a background to its proposed clinical trial in myasthenia gravis and glaucoma, has previously been reported (1). In that study and in a report from Cattell's laboratory (2), all the observed actions of di-isopropyl fluorophosphate could be attributed to its anti-cholinesterase activity. The purpose of the present investigation was to determine what toxic effects might result from the prolonged depression of the activity of tissue cholinesterases and whether the repeated administration of DFP might reveal other direct actions on the organism.

The chronic effects of DFP were studied in dogs receiving repeated injections of high doses and in dogs, monkeys and rats receiving moderate doses over prolonged periods.

I. CHRONIC INTOXICATION IN DOGS RECEIVING HIGH DOSES OF DI-ISOPROPYL FLUOROPHOSPHATE. Two dogs were given relatively large doses (0.30 and 0.50 mgm. DFP/kgm. respectively) of a 0.5% solution in peanut oil intramuscularly in alternate hind legs three times weekly for twelve weeks. Frequent blood samples were taken for the determination of serum and red cell cholinesterase activities by the method previously described (1), and external signs of DFP intoxication were carefully noted. Following the death of the animals, they were autopsied and sections were taken for histological examination.

Results. Dog A, which received the higher dosage (0.5 mgm./kgm. three times weekly) developed generalized fibrillary twitchings in the skeletal muscles, particularly those of the legs, on the eighth day following the initial injection. This condition increased in severity, becoming extreme by the seventeenth day, and persisted with occasional alleviation until death on the two hundred and third day. During the tenth week of injections the hind legs became extremely weak, a condition which proceeded to almost complete paralysis. Five weeks after the injections were stopped, some functional improvement was apparent, but complete recovery never occurred.

The first signs of muscarinic action appeared in the fourth week and consisted of dyspnea and wheezing (presumably resulting from bronchial constriction), excessive salivation, and hyperactive peristalsis. These signs were intensified by excessive activity, and after running a short distance the animal's respiration became markedly asthmatic and a temporary spastic paralysis ensued.

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Shortly afterwards dysphagia developed and the dog frequently regurgitated its food almost immediately after swallowing it, although there was no anorexia. Oddly enough, most of the muscarinic effects disappeared during the sixth week of injections and the respiration became completely normal. The second week after discontinuation of the injections there developed a constant dribbling of urine which continued until the death of the animal, and at autopsy the bladder was found to be greatly distended.

The inability to retain food persisted after the injections were stopped. The dog became extremely emaciated, and on the one hundred sixty-eighth day a fluoroscopic examination was made. When 50 cc. of a barium sulfate suspension were introduced by a tube passed just below the glottis, the esophagus was outlined as a shadow approximately 5 cm. in diameter throughout most of its length. During the fifteen minute observation period the suspension remained in the esophagus and only a minimal amount passed through the cardiac sphincter into the stomach. Most of the material was regurgitated shortly afterward. No other abnormalities were observed.

Dog A was found dead one hundred and nineteen days after the last injection. Externally it showed extreme emaciation and an absence of hair in the regions of the face, tail and legs from a persistent skin condition. Grossly the lungs showed consolidation of the upper left lobe and slight congestion of the lower lobes; the trachea contained frothy fluid. The esophagus was empty but markedly dilated from just below the larynx to the cardiac end of the stomach, confirming the fluoroscopic findings; the cardiac sphincter showed no gross abnormality. The stomach was greatly reduced in size. The remainder of the gastro-intestinal tract contained only traces of bile and was distended with gas. The urinary bladder, as already mentioned, was distended with about 200 cc. of urine. The other organs appeared normal.

Microscopically, the upper portion of the esophagus appeared normal; near the cardiac end the epithelium was partially sloughed and a moderate round cell infiltration was present. The wall was thinned and in places slightly edematous; most of the mucosal glands and their ducts were dilated and filled with mucus. The stomach showed a moderate relative thickening of the wall and occasional areas of round cell infiltration extending into the sub-mucosa. The parenchymal cells of the liver were atrophied, reflecting microscopically the loss of weight of the animal. A section of lung from the consolidated area showed the alveoli to be filled with polymorphonuclear leucocytes and fibrinous exudate. The kidney was normal excepting for a slight degree of chronic pyelitis, and the prostate showed slight inflammatory changes, more marked in the area adjacent to the urethra, the mucosa of which likewise evidenced chronic inflammation. Sections of the sciatic nerve from the areas where the injections were made showed separation of the fibers and one small area of degeneration, possibly due to trauma as no inflammatory cells were present. A section of muscle from the same region appeared completely normal.

Dog B, which received the smaller dose (0.3 mgm. DFP/kgm. three times weekly), developed the same type of dysphagia and urinary incontinence as

described above, and an even greater paralysis of the hind legs at an earlier date than dog A. The skeletal muscle fibrillation did not appear until the ninth week of injection and was not nearly as severe as in the animal receiving the higher dose. The muscarinic signs of bronchospasm and hypersalivation were not observed at any time, although there was occasional diarrhea. The animal was found dead seventy-eight days after the last injection and presented an external appearance of extreme starvation. The only abnormality found on autopsy was a marked dilation of the esophagus, which was stuffed with food from just below the glottis to the cardiac sphincter and measured about 7 cm. in diameter at the point of greatest dilatation. The walls were thin and no stricture was observed at the cardiac end.

The serum cholinesterase activity, following the original depression by the first injection of DFP, remained within fairly close limits throughout the course of injections in both dogs (table 1). The red cells exhibited a continuous fall in cholinesterase activity until very low levels were reached. This contrast is to be expected from the previously described rapid rate of regeneration of serum cholinesterase following single injections of DFP, as compared with the slow return of red cell cholinesterase at a rate comparable to that of the formation of new erythrocytes. After the injections were discontinued, the serum cholinesterase levels did not return to normal but remained around 60 per cent of the original values in both dogs. This was at the time when severe emaciation had developed and probably represented the concentration of the serum proteins as a whole. The red cell cholinesterase, on the other hand, returned at about the same rate seen in dogs following equal inhibition by single doses of DFP.

II. Chronic effects of di-isopropyl fluorophosphate in dogs, monkeys and rats receiving moderate doses over prolonged periods. Previous reports (1, 3) have shown that a fairly close parallelism exists between the tissue (i.e., brain) cholinesterase sensitivity to DFP and the dose necessary to produce certain pharmacological actions (e.g., skeletal muscle fibrillation) in different species. The dose ranges selected for the three species studied were therefore comparable to or higher than the contemplated dose range to be used in treating myasthenia gravis patients, as based on relative brain cholinesterase sensitivities. Amounts of DFP that might be absorbed systemically during the treatment of glaucoma by the ocular instillation of DFP are of a far lower order.

Procedure. Four female dogs were given intramuscular injections of DFP in peanut oil in alternate hind legs twice weekly for six months in doses of 0.05, 0.1, 0.1 and 0.3 mgm./kgm. respectively. Immediately before the first injections, and at periods thereafter of 2, 4, 8, 12, 16, 21 and 24 weeks, blood samples were taken on which the following observations and tests were conducted: red cell count, white cell count, differential count, blood sugar (4), serum protein (5), plasma non protein nitrogen (6), liver function (7) and serum and erythrocyte cholinesterase activity (1).

During this time the animals were carefully observed for overt signs of DFP intoxication. Before being sacrificed, the dogs were X rayed during the injection of 50 cc. of a barium sulfate suspension through a tube passed into the anterior portion of the esophagus. The animals were sacrificed by the intravenous injection of sodium pentobarbital and autopsies were performed. Sections for histological examination were taken from the esophagus,

TABLE 1

Effects of repeated intramuscular injections of high doses of di-isopropyl fluorophosphate on dogs. Injections given 3 × weekly for 12 weeks

DAYS SINCE INITIAL INJECTION	DOG A (0.5 MGM. DFP/KGM.)				DOG E (0.3 MGM. DFP/KGM.)			
	Ch.E. Activity		Weight	Symptoms	Ch.E. Activity		Weight	Symptoms
	Serum	RBC			Serum	RBC		
			kgm.				*kgm.*	
0	100%	100%	10.6		100%	100%	13.4	
1	27	61			29	81		
2	40	71	9.9		37	83	12.7	
3	33	44			29	74		
4	49	38	10.2		43	78	13.2	
5	34	38			33	69		
7	64	35	10.2		49	55	13.1	
8	57	21		Sl. F.	34	50		
9	49	25	10.0		45	48	12.9	
11	36	11	9.8	M. F.	39	34	12.7	
14	55	18	9.8	Sl. F.	54	28	12.7	
16	42	18	9.8	Sev. F.	43	34	12.8	
18	47	12	9.6	Sl. F.	46	28	13.2	
26	29	5		Sev. F.; Dn.	34	22		Sl. W.; An.; Coryza
35	55	14	8.8	M. F.; Sl. Dn. & Dg.	54	20	11.3	
39	43	18	9.0	M. Dn. & Dg.	47	25	11.2	
46	49	7	8.8	M. F.; Dn. absent				Sl. W.
49				*M. F.*	*50*	*12*	*10.5*	
52	62	17	8.7					
66	43	19	8.9	Sev. F. & W.	40	25	10.9	Sl. F.; M. W.
74	36	12		Sl. F., M. W.	34	29		M. F. & W.
78	56	25	8.3	Sl. F.; Sev. W.	46	49	10.9	
81	42	15	8.2		44	41	10.4	Sev. W.
*84**	53	29	8.2		42	55	10.4	
87	39	20		U. I.	46	24		
93	61	45	8.6		56	68	10.9	U. I.
99	60	58	8.9		56	64	10.9	
105		56	9.0		65	81	11.1	
109	77	76	8.4		67	80	10.9	
122	72	87	8.4	Sl. F. & W.	62	99	11.4	
138	70	107	7.5	Em.; Sev. Dg.	63	108	10.4	
152	61	103	7.3		65	118	9.8	Em.
162							7.8	Dead
173	60							
184	54	98	7.0					
203	46	74	5.7	Dead				

* Last injection.

Sl.—Slight; M.—Moderate; Sev.—Severe; F.—Fibrillation; Dn.—Dyspnea; Dg.—Dysphagia; W.—Weakness; U. I.—Urinary Incontinence; An.—Anorexia; Em.—Emaciation.

TABLE 2

Chronic effects of repeated injections of di-isopropyl fluorophosphate in dogs

All dogs injected intramuscularly 2 × weekly with the following doses of DFP in Peanut Oil: Dog O: 0.05 mgm./kgm.; Dog P: 0.10 mgm./kgm.; Dog Q: 0.10 mgm./kgm.; Dog R: 0.30 mgm./kgm.

DOG	WEIGHT	TIME SINCE INITIAL INJECTION	RED CELL COUNT	WHITE CELL COUNT	DIFFERENTIAL COUNT					BLOOD SUGAR	SERUM PROTEIN	PLASMA NPN	HEPATIC FUNCTION*	CHOLINESTERASE†	
					P M N	Lymph	Mono.	P.M.E.	P M B.					Serum	R.C.B.
	kgm.									mgm per 100 cc	per cent	mgm per 100 cc.	per cent		
O	9.3	Control	4,660,000	16,350	73	21	2	4	0	100	6.10	30.3	15	100	100
P	8.9	Control	5,510,000	19,800	86	12	0	2	0	129	6.52	25.2	10	100	100
Q	9.5	Control	5,340,000	20,700	87	6	1	6	0	129	6.38	25.0	10	100	100
R	11.6	Control	5,600,000	18,100	79	18	0	3	0	137	6.54	31.3	Trace	100	100
O	9.8	2 weeks	4,270,000	19,450	82	13	1	4	0	80.3	6.16	39.2	Trace	58	116
P	9.1	2 weeks	4,500,000	27,050	89	11	0	0	0	76.1	5.62	28.5	Trace	65	64
Q	8.2	2 weeks	5,060,000	16,400	88	11	0	1	0	79.2	5.90	20.7	Trace	66	84
R	11.5	2 weeks	5,700,000	20,300	83	17	0	0	0	86.5	6.35	30.9	0	66	56
O	10.4	4 weeks	7,150,000	17,100	69	10	2	19	0	79.3	5.32	24.3	Trace	54	111
P	9.2	4 weeks	5,310,000	29,000	80	15	3	2	0	91.3	6.41	21.7	Trace	67	78
Q	9.7	4 weeks	6,380,000	27,200	70	20	3	7	0	85.3	5.28	18.3	Trace	61	96
R	11.5	4 weeks	6,810,000	12,150	63	9	2	25	1	81.0	5.73	23.8	Trace	58	40
O	9.3	8 weeks	6,160,000	14,200	77	19	1	3	0	96	6.48	28.8	Trace	42	87
P	8.8	8 weeks	5,500,000	24,400	77	16	2	5	0	119	7.24	29.9	Trace	60	81
Q	8.0	8 weeks	6,000,000	10,900	79	16	1	4	0	93	6.59	26.2	Trace	64	95
R	12.1	8 weeks	6,130,000	19,900	63	34	0	3	0	102	6.89	28.8	Trace	53	29
O	9.1	12 weeks	5,620,000	11,200	60	26	2	12	0	104	5.87	23.4	Trace	34	116
P	8.7	12 weeks	5,280,000	13,200	72	28	0	0	0	82	7.37	22.2	Trace	58	77
Q	9.1	12 weeks	4,960,000	10,500	78	21	0	1	0	95	5.18	21.6	Trace	57	78
R	11.6	12 weeks	4,670,000	8,100	67	26	3	4	0	102	6.00	19.6	Trace	48	53
O	9.6	16 weeks	6,490,000	8,300	74	16	1	9	0	99	6.47	21.6	Trace	47	82
P	9.3	16 weeks	5,750,000	11,900	80	18	2	0	0	116	6.93	21.0	Trace	59	61
Q	10.2	16 weeks	5,590,000	8,700	68	32	0	0	0	116	6.19	26.7	Trace	51	78
R	11.8	16 weeks	6,510,000	8,300	69	30	1	0	0	111	6.47	19.3	Trace	55	39
O	10.3	21 weeks	7,020,000	16,300	72	20	2	6	0	132	5.63	19.3	0	41	81
P	9.6	21 weeks	5,720,000	11,300	86	12	2	0	0	98	5.66	28.5	Trace	49	46
Q	10.1	21 weeks	5,660,000	15,000	72	26	0	2	0	100	5.66	31.8	0	45	73
R	12.0	21 weeks	6,620,000	10,250	66	33	1	0	0	97	5.28	30.0	0	44	22
O	11.3	24 weeks	5,820,000	15,000	69	27	4	0	0	99	6.13	40.3	Trace	50	86
P	10.6	24 weeks	4,460,000	10,400	81	18	1	0	0	113	5.95	33.4	Trace	65	82.5
Q	10.5	24 weeks	5,640,000	16,700	72	28	0	0	0	94	5.63	29.6	Trace	53	69.5
R	12.0	24 weeks	5,350,000	15,100	68	32	0	0	0	94	5.89	32.5	Trace	58.5	42.5

* Reported as per cent of dye retained in serum 15 minutes after intravenous injection of 5.0 mgm. bromsulphalein/kgm.

† Reported as per cent of activity of control sample.

stomach, small intestine, large intestine, liver, kidney, ureter, urethea, bladder, lung, trachea, heart, spleen, pancreas, ovary, follopian tube, uterus, lymph node, sciatic nerve and muscle (the last two tissues were removed from the region where the DFP injections had been made). The entire brain was removed and homogenized in the Waring Blender with four times its volume of 0.025 M sodium bicarbonate and an aliquot taken for determining cholinesterase activity. Control values were obtained from the homogenates of two normal dogs similarly prepared.

Four Rhesus monkeys were given intramuscular injections of DFP in peanut oil twice weekly for four months at doses of 0.03 and 0.05 mgm. DFP/kgm. (two animals on each dose). One animal on the lower dose developed an infected ulcer on the hand and died six weeks after the start of the injections. It was replaced by another monkey (Mm) which was carried through for the full four month period. The same tests and observations were carried out on the monkeys as described above for the dogs, with the exception of the brain cholinesterase values.

Three groups of twelve young adult rats each (six males, six females) were given injections of DFP in peanut oil in alternate hind legs twice weekly at dose levels of 0.1, 0.3 and 0.5 mgm./kgm. respectively. Records of weight changes were kept, and after twelve weeks two to four animals from each group were sacrificed, blood samples taken for serum

TABLE 3

Cholinesterase activity of brains of dogs receiving repeated intramuscular injections of di-isopropyl fluorophosphate

DOG	DOSE DFP 2 × WEEKLY	CHOLINESTERASE ACTIVITY, PER CENT OF NORMAL
	mgm./kgm.	
O	0.05	60.5
P	0.1	52
Q	0.1	44.5
R	0.3	29.5

bilirubin determinations by the method of Malloy and Evelyn (8), and autopsies performed. Injections were continued in the eight remaining rats in each group for a total of twenty-four weeks. At the end of this period, four animals from each group were sacrificed by the intracardiac injection of air and their entire brains were removed and homogenized by the method previously described (1) for the determination of cholinesterase activity. Control figures obtained at that time were used here for calculating enzyme inhibition. The remaining rats were sacrificed by chloroform inhalation and their brains taken for histological examination. All animals were autopsied and the same tissues were examined histologically as in the dogs mentioned above.

Results. A. Dogs. Throughout the six-month period during which DFP was given twice weekly, all four dogs remained in good general health with the exception of the development of an inflammatory skin condition and a resulting loss of hair over a large area of the body. Most of the other dogs in the colony were similarly affected, but shortly after treatment was instituted by dipping in a lime-sulfur bath, the inflammation disappeared and a normal growth of hair reappeared in the denuded areas. The only sign of DFP intoxication observed in these dogs was skeletal muscle fibrillation which occurred sporadically in all four, usually appearing a few hours following an injection and persisting for a few hours to several days. At the time they were sacrificed all appeared normal

TABLE 4

Chronic effects of repeated injections of di-isopropyl fluorophosphate on rhesus monkeys

All monkeys injected intramuscularly 2 × weekly with the following doses of DFP in Peanut Oil: Monkey Im· 0.03 mgm./kgm.; Monkey Mm: 0.03 mgm./kgm.; Monkey Km: 0.05 mgm./kgm.; Monkey Lm: 0.05 mgm./kgm.

MONKEY	WEIGHT	TIME SINCE INITIAL INJECTION	RED CELL COUNT	WHITE CELL COUNT	DIFFERENTIAL COUNT					BLOOD SUGAR	SERUM PROTEIN	PLASMA NPN	HEPATIC* FUNCTION	CHOLINESTERASE†	
					P M N	Lymph	Mono	P M E	P M B					Serum	R B C.
	kgm									*mgm per 100 cc.*	*per cent*	*mgm per 100 cc*	*per cent*		
Im	2.50	Control	5,310,000	12,600	51	45	0	4	0	145	6 85	33.3	35	100	100
Mm	2 95	Control	4,730,000	22,700	63	37	0	0	0	137	5 68	38.2	25	100	100
Km	2.95	Control	5,010,000	12,500	35	61	3	1	0	139	7.44	37.1	15	100	100
Lm	3.06	Control	5,320,000	11,000	34	60	4	1	1	141	7 94	33 4	25	100	100
Im	2.72	2 week	4,360,000	13,800	64	33	0	3	0	159	6 88	30.6	15	6	20
Mm															
Km	2.94	2 weeks	5,510,000	8,200	49	41	4	6	0	125	7 44	34.4	30	29	7.5
Lm	2.94	2 weeks	6,110,000	10,700	33	63	1	3	0	122	7 19	32 9	15	31	7.3
Im	2.85	4 weeks	4,650,000	13,800	54	42	2	1	1	115	6.62	33.3	15	43	13
Mm	2.50	4 weeks	5,180,000	11,400	52	46	0	2	0	192	6 13	32 8	Trace	42	12 5
Km	2 90	4 weeks	4,480,000	22,450	77	23	0	0	0	139	6 62	32 6	25	0	3
Lm	2.94	4 weeks	4,630,000	13,750	58	35	2	5	0	147	6 93	30.6	25	36	9
Im	2.72	8 weeks	3,640,000	19,800	54	45	1	0	0	196	6 02	42.9	Trace	26	4
Mm	2.51	8 weeks	5,280,000	19,100	80	19	0	1	0	91	6 24	27 3	30	30	13
Km	2 53	8 weeks	5,500,000	16,100	35	65	0	0	0	144	5.66	42.7	10	0	0
Lm	2.81	8 weeks	4,300,000	9,900	40	60	0	0	0	160	5 83	30 2	Trace	21	6
Im	2.41	12 weeks	5,390,000	7,800	84	14	2	0	0	110	6 04	44.5	15	32	9.5
Mm	2.80	12 weeks	5,340,000	20,350	59	37	2	2	0	110	6 17	26.8	15	61	13
Km	2.50	12 weeks	6,510,000	9,500	62	38	0	0	0	107	6 23	33 4	10	44	2
Lm	2.86	12 weeks	4,480,000	11,900	63	35	2	0	0	146	6 95	37.0	15	40	5
Im	2 00	16 weeks	4,790,000	16,000	72	28	0	0	0	121	5 40	39.3	20	40	11
Mm	2 60	16 weeks	5,160,000	18,700	82	17	0	1	0	143	6 16	47 7	Trace	51	28
Km	2 28	16 weeks	4,270,000	14,000	83	14	1	2	0	91	5 49	27 9	15	39	5.5
Lm‡															

* Reported as per cent of dye retained in serum 15 minutes after intravenous injection of 5 0 mgm bromsulphalein/kgm

† Reported as per cent activity of control sample

‡ Found dead 96th day after initial injection

and had gained in weight since the beginning of the injections. Figures obtained from the blood studies (table 2), excluding the cholinesterase values, remained within the limits of normal variation with the exceptions here noted. The relatively high control values for blood sugar are attributed to the excitement and struggling which occurred at the beginning of the study when the dogs were tied

to the animal boards. During subsequent tests they had become sufficiently trained that they remained relatively quiet. The high white cell counts, particularly for dog P, occurred at the time when the inflammatory condition of the skin was especially severe and secondary infection had set in. With the treatment and subsequent disappearance of the disease, the counts returned to normal.

TABLE 5

Weight changes in rats receiving repeated intramuscular injections of di-isopropyl fluorophosphate in oil

Each figure represents the average weight of the survivors of an original group of twelve rats (six males, six females).

DFP injections made twice weekly in doses indicated.

WEEKS SINCE INITIAL INJECTION	GROUP A 0.1 MGM./KGM.	GROUP B 0.3 MGM./KGM.	GROUP C 0.5 MGM./KGM.	REMARKS
	grams	*grams*	*grams*	
0	217.6	223.3	210.2	
1	225.7	229.8	212.4	
2	237.7	217.3	214.8	Rat # 4, male, Group A, found dead.
3	244.4	242.7	224.3	
4	248.8	250.7	227.8	
5	253.3	245.2	231.4	
6	259.9	256.9	233.4	
7	257.6	259.3	230.8	
8	263.4	269.0	245.4	Rat # 5, male, Group A, found dead (pneumonia).
9	262.1	270.9	245.1	
10	268.6	278.3	249.5	
11	270.2	281.9	252.8	Rat # 27, male, Group C, found dead.
12	288.3	286.8	265.6	
13	290.5	285.8	264.0	Rats # 11, 12 (Group A), 17, 18, 23, 24 (Group B), 30, 35, 36 (Group C) sacrificed and autopsied.
14	288.5	264.0	265.4	
15	290.5	291.3	270.0	
16	292.9	295.8	274.1	
17	293.5	300.5	278.1	
18	299.5	304.4	276.7	Rat #34, female, Group C, found dead.
19	307.0	310.0	288.3	
20	294.4	298.8	272.3	
21	295.0	299.0	281.9	
22	295.9	297.6	283.4	Rat # 10, female, Group A, found dead. All survivors sacrificed.
23	292.4	294.8	286.4	
24	307.9	307.8	288.7	
Increase since initial injection	90.3	84.5	78.5	

The X-ray plates of dogs O, Q, and R revealed esophagi of normal size and activity from which the barium sulfate suspension passed through the cardiac end into the stomach with no abnormal delay. In dog P, the plate taken during the first injection disclosed that the suspension had remained in the esophagus, which was slightly enlarged throughout its length. In this animal observations were repeated thirty minutes later, at which time the originally injected suspen-

sion had passed into the stomach but the second injection remained temporarily in the esophagus as had the first. The evidence of cardiospasm was confirmed at autopsy.

Autopsy findings. Dog P regurgitated a large amount of partially digested milk just before respiration ceased following the sodium pentobarbital injection. More was found in the stomach and esophagus. The latter organ was dilated to about 3 cm. in diameter along its entire length, but there was no gross evidence of abnormality of the cardiac sphincter. This animal's thymus was slightly larger

TABLE 6

Cholinesterase activity of brains of rats receiving repeated intramuscular injections of di-isopropyl fluorophosphate

RAT NO	DOSE DFP 2X WEEKLY	CHOLINESTERASE ACTIVITY, PER CENT OF NORMAL
	mgm./kgm.	
1	0 1	72.5
2	0 1	64
7	0 1	61.5
8	0 1	63 5
Average		65.4
13	0 3	26
14	0 3	31
19	0 3	25
20	0 3	31
Average		28.3
25	0 5	18
26	0 5	20.5
31	0 5	25
32	0 5	22
Average		21 4

than normal. The kidney of dog O exhibited a slight degree of cloudy swelling. There was an appearance of questionable degeneration of ganglion cells of Meissner's and Auerbach's plexi in the lower part of the esophagus of dog R. The spleen in all four dogs was slightly enlarged and had rounded edges and soft pulp. Microscopically, these organs were slightly to moderately congested and contained large amounts of pigment which appeared to be hemosiderin. All other organs were grossly and microscopically normal. Brain cholinesterase values are presented in table 3.

B Monkeys (Macaca mulatta). The animals remained in good health during the first twelve weeks of injections, after which monkeys Im, Km, and Lm began to lose weight and shortly thereafter developed bronchopneumonia. Up to this time the blood chemistry and formed elements had remained essentially normal,

allowing for the wide variation in the white cell count that is routinely found in this species (table 4). As in the dogs, skeletal muscle fibrillation was seen occasionally but was not persistent; no other signs of DFP intoxication appeared. With the onset of bronchopneumonia the three animals became weak and emaciated, their appetites were poor and the serum protein concentrations fell. Monkey Lm was found dead on the 96th day and an autopsy was performed. Monkey Mm maintained a healthy condition throughout the sixteen week period.

Prior to being sacrificed, the three surviving animals were fluoroscoped during the injection of 30 cc. of a barium sulfate suspension into the esophagus through a tube extending a few centimeters below the larynx. In each case the outline of the esophagus presented no abnormalities and there was no delay in the passage of the material into the stomach.

No gross abnormalities were found at autopsy in this group. Microscopically, numerous small encysted oil droplets were seen in the muscle at the site of the injections in all animals, but the surrounding tissues and adjacent nerves showed no signs of inflammation. The lungs of monkeys Im, Km and Lm exhibited varying degress of bronchopneumonia. The esophagus of monkey Im contained numerous encapsulated filarial worms and eggs within the epithelium. Several small round areas of calcification were seen in the adrenal cortex of monkey Mm. All other organs appeared normal.

C. Rats. With the exception of five rats that died out of the total group of thirty-six, the animals remained in a condition of good general health. Three of those found dead were in group A (0.1 mgm. DFP/kgm. 2 × weekly) and two in group C (0.5 mgm. DFP/kgm. 2 × weekly). Autolysis had proceeded too far to permit any definite conclusions as to the cause of death in these animals from autopsies, but in three of them signs of pneumonia were noted prior to death. The rats receiving the highest dose (group C) were the only ones in which the development of skeletal muscle fibrillation was seen and this occurred only transiently for a few hours following injections. These animals also occasionally showed diarrhea. All three groups increased in weight during the experimental period (table 5) by amounts inversely proportional to the doses received, but the significance of the differences is questionable. The increased gastro-intestinal activity of the animals on the highest dose probably interfered with the absorption of food to some extent.

The nine rats sacrificed on the eighty-third day all gave negative results for serum bilirubin.

The remaining rats were sacrificed three or four days after the forty-eighth injection. Cholinesterase determinations on the brains of four rats from each group yielded average values of 65.5, 28.3, and 21.4 per cent of normal for groups A, B, and C respectively. Individual values are given in table 6. Besides the pathological changes listed individually below, all the animals exhibited encapsulated oil droplets in the leg muscles.

AUTOPSY FINDINGS. *Group A.* Rat #1. Male. All organs grossly and microscopically normal.

Rat #2. Male. Small areas of atalectasis in upper lobes of both lungs, microscopically

showing signs of old lobular pneumonia. All other organs grossly and microscopically normal.

Rat #3. Male. All organs grossly and microscopically normal.

Rat #6. Male. Spleen slightly congested. All other organs grossly and microscopically normal.

Rat #7. Female. Slight congestion of spleen and of zona fasciculata of adrenal cortex. All other organs grossly and microscopically normal.

Rat #8. Female. Large area of atalectasis in left lower lobe. All other organs grossly and microscopically normal.

Rat #9. Female. Slight bronchopneumonia. Spleen slightly congested. All other organs grossly and microscopically normal.

Rat #11. Female. Adrenal medulla markedly congested. Moderate bronchopneumonia. No other abnormalities.

Rat #12. Female. No abnormalities.

Group B. Rat #13. Male. Right lung collapsed; small white nodule approximately the size of a pea at base of middle lobe, surrounded by smaller similar nodules, microscopically seen to be an encysted organizing abscesses. Surrounding area shows moderate ataleetasis, emphysema, and organizing pneumonia. All other organs grossly and microscipically normal.

Rat #14. Male. Small area of atalectasis in right upper lobe. Spleen moderately congested. All other organs grossly and microscopically normal.

Rat #15. Male. Spleen and kidneys slightly congested. All other organs grossly and microscopically normal.

Rat #16. Male. Spleen and lungs slightly congested. All other organs grossly and microscopically normal.

Rat #17. Male. Tracheitis and slight bronchopneumonia. No other abnormalities.

Rat #18. Male. Spleen slightly congested. Slight bronchopneumonia. No other abnormalities.

Rat #19. Female. Slight bronchopneumonia. All other organs grossly and microscopically normal.

Rat #20. Female. Large area of atalectasis in right middle lobe. All organs grossly and microscopically normal.

Rat #21. Female. Lungs and spleen slightly congested. All other organs grossly and microscopically normal.

Rat #22. Female. Slight bronchopneumonia. Small area of degeneration in cerebral cortex. Spleen slightly congested. All other organs grossly and microscopically normal.

Rat #23. Female. Few small areas of leucocytic infiltration around the vessels of the renal cortex. Spleen slightly congested. No other abnormalities.

Rat #24. Female. No abnormalities.

Group C. Rat #25. Male. Lung shows small areas of emphysema and organizing pneumonia. All other organs grossly and microscopically normal.

Rat #26. Male. Area of hemorrhage and congestion at apex of one lower lobe. Spleen moderately congested. All other organs grossly and microscopically normal.

Rat #28. Male. Slight bronchopneumonia. Spleen slightly congested. All other organs grossly and microscopically normal.

Rat #29. Male. Slight bronchopneumonia. All other organs grossly and microscopically normal.

Rat #30. Male. Spleen moderately congested. No other abnormalities.

Rat #31. Female. Spleen and lungs slightly congested. All other organs grossly and microscopically normal.

Rat #32. Female. Spleen moderately congested. Hemorrhagic area in zona glomerulosa of adrenal cortex. All other organs grossly and microscopically normal.

Rat #33. Female. Spleen slightly congested. All other organs grossly and microscopically normal.

Rat #35. Female. Spleen slightly congested. No other abnormalities.

Rat #36. Small area of pericarditis at apex of heart. Pressure atrophy in muscle fibers adjacent to encapsulated oil droplets. No other abnormalities.

Discussion. The above observations indicate that DFP possesses no outstanding toxic actions which cannot be directly attributed to its primary effect on cholinesterase. Moreover it was necessary to inhibit cholinesterase to an extreme degree over a long period of time before reduced cholinesterase activity produced functional changes which resulted in death. It is of interest that in dogs the functional failure of the cardiac sphincter was the most outstanding physiological defect.

The production of chronic cardiospasm in dogs A, E, and P by the repeated administration of an anticholinesterase and therefore cholinergic agent appears paradoxical inasmuch as it greatly resembles the condition produced in dogs by sectioning the vagi (9). However, in a recent paper Lehmann (10) has shown that the vagus supplies cholinergic fibers to the cardia, stimulation of which causes cardiospasm. His work confirms the earlier suggestion that the inhibition of the cardiac sphincter following vagal stimulation is the result of an intrinsic reflex produced by the concomitant contraction of the upper part of the esophagus. It is not unlikely therefore that the presence of high concentrations of acetylcholine over a prolonged period could result in a predominance of cardiotonic action. In addition, the epinephrine or sympathin produced by the action of DFP on the adrenal glands and autonomic ganglia would augment this effect, as sympathetic stimulation is also known to cause contraction of the cardia (9).

The urinary incontinence might have resulted from augmentation by DFP of the impulses from the cholinergic pelvic nerves, which produce contraction of the detrusor and inhibition of the trigone muscles of the bladder (11). Unexplained by this supposition is the autopsy finding of a distended bladder in dog A.

The brain cholinesterase values shown in tables III and VI confirm the suggestion of previous findings (1, 3) that an animal can function almost completely normally when this enzyme is present at approximately 20 per cent of the normal activity over a prolonged period. It is interesting to consider Gesell's (12) theory of the integration of nervous activity through the effect of intracellular cH on cholinesterase in the light of this situation. In the original work of Glick (13), upon which the theory is based, cholinesterase was shown at pH 6.0 to have approximately twenty per cent of the activity it possessed at pH 7.4. However, these measurements were made at 25 degrees centigrade on human serum cholinesterase, which is now known to be an entirely different enzyme from that found in nervous tissue (14). Moreover, it is questionable whether such wide variations in intracellular pH can occur physiologically, and measurements of such and their interpretation present many difficulties. The use of DFP to inactivate cholinesterase irreversibly offers a new approach to this problem. The rapid regeneration of brain cholinesterase from low and apparently critical values to a "functionally normal" amount is inferred from the fact that the rats which received 48 injections of 0.5 mgm./kgm. (group C) had an average brain cholinesterase value of 21.4 per cent three days after the last injection, whereas a

single injection of 1.0 mgm./kgm. produces a fall to approximately 10 per cent of the normal value (1).

The lack of direct significance of serum and red cell cholinesterase values in regard to physiological functioning has already been shown (1, 2, 3), although such measurements can be used to determine absorption rates following the administration of DFP by various routes.

Bronchopneumonia is by no means a rare disease in laboratory monkeys, but the possibility that the prolonged subjection to sporadic nicotinic and muscarinic effects of DFP played a predisposing rôle cannot be overlooked. An increased amount of tracheal secretion plus the poorly-developed cough reflex in this species were undoubtedly associated factors. Of less significance is the frequent microscopic finding of bronchopneumonia and alatectasis in the rats. Ratcliffe (15) states that at one year about 75% of these animals show such changes.

The explanation for the hind leg paralysis seen in dogs A and B awaits more direct information concerning the effects of DFP on nerve impulse conduction and transmission. This condition followed only after a long period of nicotinic stimulation and was already irreversible when the administration of DFP was stopped. That the paralysis was the result of the effects of the prolonged inhibition of cholinesterase on peripheral myoneural conduction would appear likely. Further investigation of this phenomenon is being undertaken at present.

SUMMARY AND CONCLUSIONS

1. Cardiospasm, hind leg paralysis and urinary incontinance resulted in dogs from the repeated administration of high doses of di-isopropyl fluorophosphate. Monkeys receiving moderate doses developed a predisposition to bronchopneumonia.

2. The formed elements of the blood, blood sugar, serum protein, plasma non-protein-nitrogen and hepatic function were not directly affected in dogs and monkeys receiving moderate doses of di-isopropyl fluorophosphate over prolonged periods.

3. No toxic effects were observed in rats following the chronic administration of di-isopropyl fluorophosphate.

We wish to express our appreciation to Dr. Ralph Ferguson, of the Pathology Section, for valuable aid of the diagnosis of the gross and microscopic autopsy findings. We are also indebted to Mrs. Marion Freeman, Mrs. Ethol S. Koelle and Lt. Arthur Dziemian for their help in the blood morphology studies, chemical analyses and X-ray photography.

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SUPPLEMENT TO
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Contents

THE SYMPOSIUM ON THE PHARMACOLOGY OF DITHIOLS

In order to make available the research work which is the basis for the therapeutic use of BAL, a committee, consisting of representatives of the various groups which had taken part in the BAL study, was appointed to plan and carry out the publication of the experimental data.

Under the chairmanship of Dr. Warfield T. Longcope, the committee decided that papers on the fundamental work on BAL be selected and divided into three groups, those dealing with biochemistry, those dealing with toxicology, pharmacology and experimental therapeutics, and those dealing with clinical applications. For publication media the *British Biochemical Journal*, the JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS and the *Journal of Clinical Investigation* were considered especially suitable and it was proposed that the papers selected be published in the form of a symposium, distributed among these three journals.

The proposal submitted by Dr. Longcope was given full consideration by the Editorial Board of the JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS and it was agreed that the papers on the pharmacology of BAL be printed in this JOURNAL. The papers submitted have been reviewed and arranged in proper order and make up a supplementary issue of the current volume.

BRITISH ANTI-LEWISITE (BAL)

Among the many compounds developed under the impetus of World War II, BAL is of outstanding interest both from the standpoint of its practical application and because of the light it throws on the mechanism of action of salts of the heavy metals. In 1939, as part of a program of war research, investigations were initiated in Peters' Laboratory at Oxford which led to the demonstration of the effectiveness of the dithiols against the toxic action of arsenic in biological systems. The problem has since been actively pursued in both England and this country. The British work has been reviewed by Peters, Stocken and Thompson in *Nature* (November 24, 1945) and the American work by Waters and Stock in *Science* (December 14, 1945).

The development of the dithiols as protective agents against poisoning by metal salts was based upon the earlier fundamental contributions of Voegtlin and his associates (1923) in which it was shown that various monothiols such as reduced glutathione and thioglycollic acid counteract the toxic actions of arsenoxide in both trypanosomes and mammalian species. The mechanism of this protection, since supported by evidence from several laboratories, is believed to be based upon the availability of additional SH groups which compete for the arsenic which would otherwise combine with the body proteins. It was shown by the British investigators in *in vitro* studies that sodium arsenite and lewisite had a strong inhibitory action on certain enzymes, particularly the pyruvate-oxidase system. The monothiols were effective in protecting this system when the arsenic was employed in the form of aromatic arsenoxides but was of no value against lewisite. This fact led to the preparation and testing of several dithiols on the theory that they might be more effective through the formation of relatively stable ring compounds with lewisite and other trivalent arsenicals. One of these compounds, 1–2 dimercaptopropanol, subsequently coded BAL (British Anti-Lewisite) was selected on the basis of its physical and chemical properties as likely to be the most effective in affording protection against the toxic action of arsenic in biological systems. BAL has the following structural formula:

$$\begin{array}{ccccc} C & - & C & - & C \\ | & & | & & | \\ SH & & SH & & OH \end{array}$$

BAL is a colorless liquid with a specific gravity of 1.21 and is soluble in water to the extent of 6 per cent. In common with other mercaptans it has a strong skunk-like odor. BAL reacts with lewisite and other arsenicals to form insoluble, non-dissociable thioarsenate complexes. In this form arsenic is relatively nontoxic to mammals and does not inhibit the pyruvate-oxidase system. Further, for a considerable period after the addition of lewisite or other arsenicals the inhibition may be reversed by the addition of BAL.

Not only has a large amount of work been done on the pharmacology and toxicity of BAL and related dithiols, some of which is described in the present series of papers, but the investigations have been extended to man where its

effectiveness in the treatment of arsenic and mercury poisoning has been demonstrated.

In the recommended dosage of 2.5 mg./Kg. of body weight at 4 hour intervals, given as a 10 per cent solution of BAL in oil by intra-musuclar injection, there have been very few reactions and these are of a minor nature. Occasional patients complain of nausea, generalized aches and pains or a burning sensation in the mouth or eyes. The blood pressure is frequently raised and rarely there may be a sense of constriction in the throat and chest. These symptoms do not last more than 30 to 60 minutes and no cumulative toxicity results from repeated injections at 4 hour intervals.

BAL is effective by local application in decontaminating the skin and preventing the toxic effects of lewisite. By intra-muscular injections excellent results have been obtained in certain of the serious toxic reactions following mapharsen, especially toxic encephalopathy and dermatitis. However, it is of doubtful value in blood dyscrasias and jaundice due to arsenic. While difficult to evaluate, the results to date indicate that BAL is remarkably effective in mercury poisoning, all of a consecutive series of thirty patients having recovered under this treatment, some of whom otherwise would not have been expected to survive.

PHARMACOLOGY OF SEVERAL DITHIOLS RELATED TO 2,3-DIMERCAPTOPROPANOL (BAL)[1]

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The present paper deals with the results of pharmacologic investigations of nine additional dithiols which were submitted to us in the course of the cooperative program of the several government agencies. The extent of the study of the various compounds, was not equal. Some were investigated in considerable detail. In the case of others only such matters were explored as seemed necessary in relation to studies in progress in other cooperating laboratories. The study of some was interrupted when information became available indicating that the compounds lacked promise. Table 1 lists the compounds studied and their partial formulas.

2,3-DIMERCAPTOPROPYL ETHYL ETHER (NDR-293). *Cats—general effects—intravenous injection.* For intravenous injection a solution of NDR-293 in thiodiglycol and ethanol was used. This solution contained approximately 50 per cent ethanol, 20 per cent thiodiglycol and 4 per cent NDR-293 by volume. The doses are expressed in mM calculated from a specific gravity of 1.05 and molecular weight of 152.3. A dose of 0.1 cc. of thiodiglycol per Kg. given intravenously for control in one cat caused fleeting slight ataxia without any other symptoms.

The general systemic effects of intravenous injections of NDR-293 in unanesthetized animals were studied in 19 cats. The doses ranged from 0.007 mM (0.001 cc.) to 0.41 mM (0.06 cc.) per Kg.[3] The smallest dose used produced no immediate effects, but by the following day there were mild symptoms of poisoning, namely, slight depression, some muscular spasticity, weakness, impaired reflexes and anorexia. Even with this very small dose the duration of action was very long, and signs of illness were still present 37 days later when the animal was sacrificed.

A dose five times as large produced more severe effects and a picture of poisoning fairly characteristic of all the larger doses. Three phases were distinguishable in the course of the poisoning. There was a brief period of depression, sometimes with signs of collapse, ataxia and weakness. This was followed by a protracted period of convulsive hyperexcitability and hyperactivity. Among the symptoms the following were included: lacrimation, blepharospasm, dilated pupils (react to light), foamy salivation, vomiting, nystagmus, urination, changes in

[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

[3] For the sake of brevity, "per Kg." will be omitted since all doses are state in terms of body weight.

TABLE 1

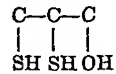
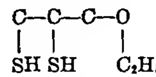
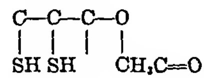
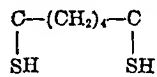
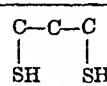
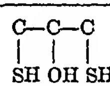
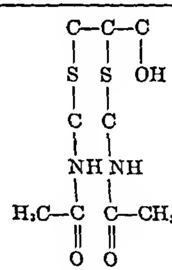
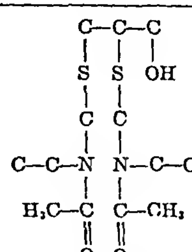
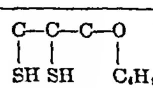
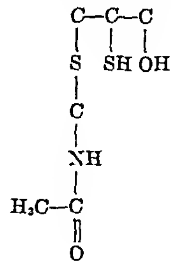
NAME	FORMULA	MOL. WT.
2,3-dimercaptopropanol (BAL) (NDR-133)		124.2
2,3-dimercaptopropyl ethyl ether (NDR-293)		152.3
2,3-dimercaptopropyl acetate (NDR-230)		166.2
Hexanedithiol-1,6 (NDR-139)		150.3
Propanedithiol-1,3 (NDR-132)		108.2
1,3-dithioglycerol (NDR-397)		124.2
Bis-S(acetamidomethyl) ether of BAL (NDR-406)		266.4
Bis-S(N-ethylacetamidomethyl) ether of BAL (NDR-407)		322.5
2,3-dimercaptopropyl butyl ether (NDR-420)		180.3

TABLE 1—*Concluded*

NAME	FORMULA	MOL. WT
Mono-S(acetamidomethyl) ether of BAL (NDR-475)		195.2

affective state, the animal growling and gnashing the teeth but offering no resistance to painful stimuli, loss of initiative, awkward gait, impaired placement reflexes, abnormal postures, the animal lying with the hind limbs flexed and fore limbs extended with the head in marked extension. The behavior suggested decerebration. There was marked stimulation of respiration with panting. After the larger doses initial temporary paralysis of respiration was sometimes produced.

On the day following the injection the signs of hyperactivity usually subsided and symptoms of diminished activity appeared which lasted for many days or weeks. In a few cases there was a tendency to slow recovery but most animals showed little tendency to improvement throughout the course. In this period spontaneous activity was almost absent and the animals seemed incapable of effective reaction to the environment or to specific stimuli. They tended to remain in whatever position they were placed, in a catatonic-like state, some assuming a fetal posture remaining curled up for may days until death. They sometimes stood immobile, staring directly ahead for long periods. Anorexia was often complete, the animal refusing food or water and losing as much as one-fourth to one-third of the body weight until death with emaciation in two weeks or longer.

The effect on the eyes, namely, lacrimation and blepharospasm, is much less pronounced than in the case of BAL. The effects on the central nervous system dominate the picture of poisoning, whereas in the case of BAL, the circulatory effects are outstanding. Also in the case of BAL the course of poisoning is essentially acute, while in the case of NDR-293 a chronic and, for the most part, an irreversible damage of the central nervous system takes place.

The onset of the symptoms following the intravenous injection of the larger doses was almost immediate. The course was protracted over periods of two weeks or longer, usually terminating in death. One animal showed complete recovery after 4 days, two others after 21 and 28 days, respectively.

An acutely fatal outcome was observed in only one animal, death occurring with respiratory failure within 3 minutes after a dose 60 times that which causes

mild symptoms. It is noteworthy that for NDR-293 the action causing acute death is relatively feeble by comparison with that action which gives rise to chronic poisoning; thus a dose 40 times that which causes mild poisoning does not kill quickly but produces a state of chronic poisoning lasting weeks.

We do not have sufficient experiments to relate the dose to the duration of the course of fatal poisoning. The indications from 4 cases in which the course terminated spontaneously are that the size of the dose has little influence on the length of life provided the animal does not die from the acute effects. In this group of 4 animals the survival periods were essentially the same in the animal that received 0.035 mM as in those which received two and four times as much. It may well be that death in the chronic poisoning is not the direct result of injury of the central nervous system but of the inanition as a consequence of the poisoning.

The inanition was of a type, however, which could not be prevented by administering a high caloric diet by stomach tube. Each of 4 cats was fed daily in this manner. The period of poisoning was somewhat prolonged, although symptoms were not significantly changed. The forcing of food resulted in vomiting and the loss of weight progressed, in some cases in a manner similar to those animals which were not so fed although the loss of weight seemed to have been retarded in other cases.

Cats—general effects—intramuscular injection. The effect of the intramuscular injection of NDR-293 was studied in 12 cats. There were 15 doses. In these experiments the drug was injected undiluted. The doses varied from 0.07 mM (0.01 cc.) to 0.34 mM (0.05 cc.). Qualitatively the symptoms were similar to those after the intravenous injections. The onset of effects was delayed for variable periods of time from 4 to 75 minutes. In one case there was a delay of about 19 hours after detectable effects. The smallest dose used, 0.07 mM, caused mild symptoms with recovery in 2 days. The toxicity by intramuscular injection showed much wider variation than by intravenous injection, suggesting irregular absorption. It is not feasible to obtain a ratio of the toxicity by intramuscular and intravenous injection because of the irregular effects following the intramuscular doses. In one case, a dose by intramuscular injection caused less effect than $\frac{1}{20}$ of that dose by intravenous injection in another animal. However, in one animal a dose of 0.14 mM intramuscularly produced no effects, while in another a similar dose produced severe poisoning in about 10 minutes with death in 14 days, an effect which is indistinguishable from that of a similar dose by intravenous injection.

The area of the muscle in which the drug was injected was excised and examined grossly and histologically in 5 animals. The agent produced necrosis of the muscle with yellowish discoloration and yielded a faint odor of NDR-293.

Cats—general effects—cutaneous application. In each of 4 cats the hair was clipped close to the skin on the ventral side of the abdomen and washed. The agent was applied undiluted to an area of skin, rubbed, and allowed to penetrate. The doses applied varied from 0.14 mM to 1.38 mM. The agent produced a local effect, a mild erythema followed by an edematous wheal of moderate sever-

ity within several minutes. Local damage to the capillaries of the skin was also shown by the observation that the area of application developed blue coloration after an intravenous injection of Evans blue (T-1824). All local effects disappeared within a day or two. The results also show that systemic absorption occurs after cutaneous application of NDR-293, symptoms appearing in from 15 to 45 minutes. Fairly marked symptoms were produced with poisoning lasting 11 days. Doses of from 20 to 40 times those which cause mild symptoms by intravenous injection, produced, by cutaneous application, little or no effect. The dose required for systemic effects by cutaneous administration lay in the range of 100 times the intravenous dose.

Cats—cardiovascular dynamics. The circulatory changes produced by NDR-293 were investigated in 17 cats. The drug was injected intravenously (doses 0.07 to 0.21 mM) in most cases in the form of the solution previously described for intravenous injection. In the 4 cases in which it was given intramuscularly

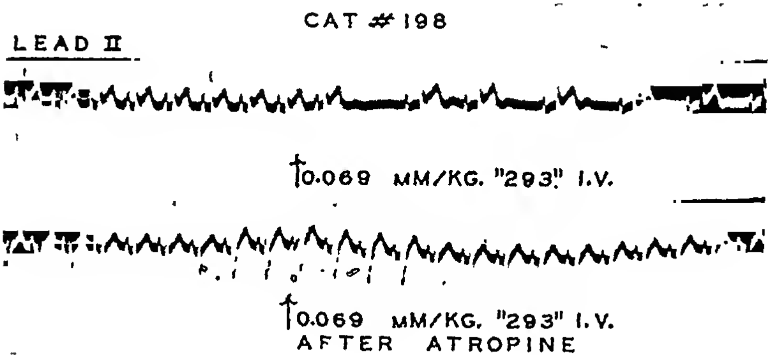


FIG. 1

(doses 0.07 to 0.35 mM) it was used undiluted. The following systems were studied: the carotid blood pressure, the portal vein pressure, the femoral or iliac vein pressure, the peripheral resistance by the Bartlett technic (1), the peripheral resistance by perfusion of the isolated leg, the cervical lymph flow, and the heart by the electrocardiogram.

Toxic doses of NDR-293 by intravenous injection caused an abrupt fall of the arterial blood pressure which often reached shock levels. In most cases the blood pressure showed a tendency to fairly prompt recovery.

There was a marked slowing of the heart which was prevented by atropine. This is shown in the electrocardiogram (fig. 1). The fall of the blood pressure was not prevented by atropine or carotid sinus denervation.

Simultaneously with the fall of blood pressure there occurred a rise of the portal pressure and a rise of the systemic venous pressure, the latter tending subsequently to fall off somewhat below the control.

Types of effects on the arterial blood pressure are reproduced in figures 2, 3, and 4. That in figure 2 is typical of the usual response, namely, an abrupt fall with rapid recovery. Figure 4 shows a vasopressor effect obtained with small doses; although the dose in this case was fairly large, it had the effect of small doses

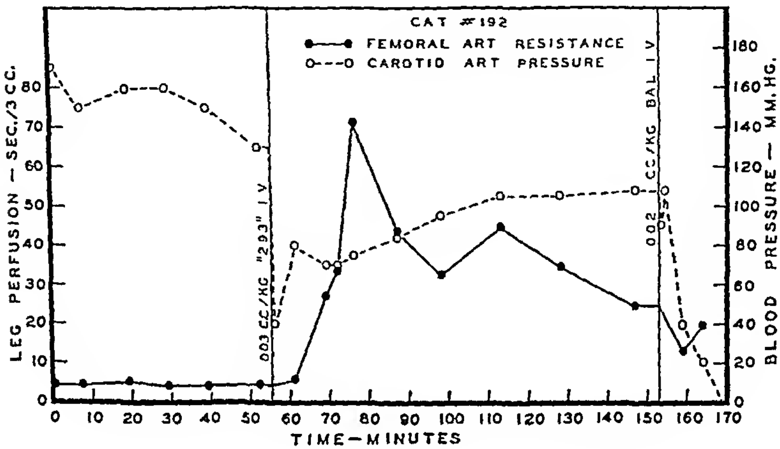


FIG. 2

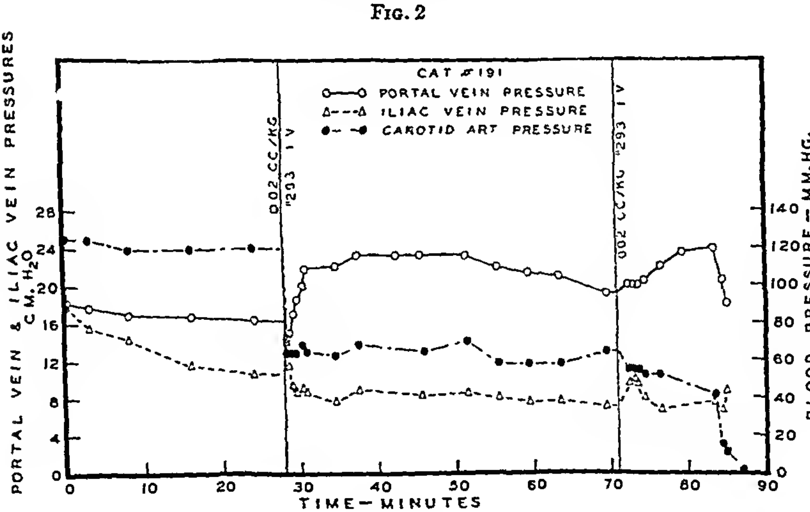


FIG. 3

because it was made intra-arterially and intense local constriction prevented free access to the circulation.

The marked rise of portal pressure is shown in figure 3 which also demonstrates the type of effect on the systemic venous pressure.

A sustained increase in peripheral resistance in the intact leg is shown in figures 3 and 4. A similar constrictor and irreversible effect in the isolated perfused leg was reported in a previous study (2).

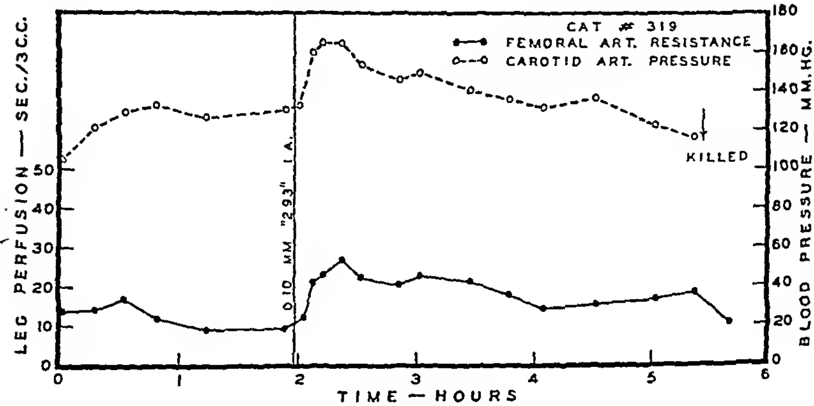


FIG. 4

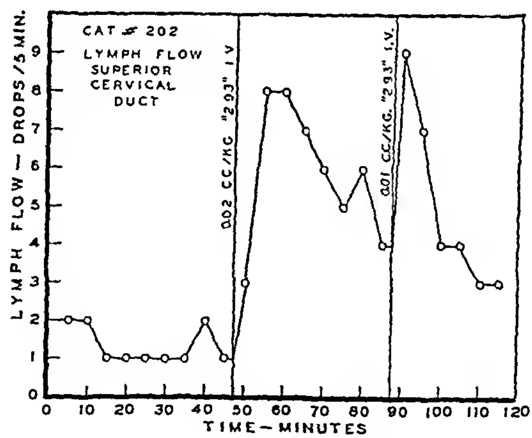


FIG. 5

Figure 5 shows a marked increase in cervical lymph flow. This is in all probability due to the increased capillary permeability shown more directly by the results of the experiments with Evans blue (T-1824) described below.

The foregoing actions on the circulation (arteriolar constriction, capillary paralysis, predominantly vasodepressor effect and shock) are in general similar to those of BAL with some modifications in design. BAL does not cause the vagal slowing of the heart. Also in the case of BAL, the arterial blood pressure continues to fall, and the increased peripheral resistance is reversible (2).

Cats—capillaries. Each of 6 cats received an intravenous injection of 5 mg. of Evans blue (T-1824). The NDR-293 was then administered intravenously in 5 and applied to the skin in 1. The animal was observed for changes in the bluish coloration in the tissues as evidence of the escape of the dye through the capillary walls. The application of 1.38 mM NDR-293 to the skin gave rise to intensely blue color in that area indicating local damage of the capillaries. The intravenous injection of doses of 0.14 to 0.28 mM in some cases strongly intensified the bluish coloration of the skin and mucous membranes. It began within less than 10 minutes after the dithiol was injected. As in the case of BAL, simultaneous determinations of the hematocrit showed hemoconcentration, in contrast to the dye in the blood which failed to concentrate. This fact, as well as the blue coloration of the tissues, indicates that NDR-293 increases the permeability of the capillaries.

Cats—brain pathology. Gross examination was made post-mortem in almost all animals. No abnormalities in the heart, lungs, kidneys, or other viscera were observed with sufficient uniformity to be ascribed to the direct action of the drug. Dehydration and loss of body fat were present in those suffering with malnutrition. The brain was examined grossly in a majority of animals and sections were stained for histological examination. Extensive degeneration in cerebral cortical, and cerebellar cells was present. In view of the fact, however, that prolonged poisoning by NDR-293 resulted in malnutrition and inanition, the significance of these findings remained in doubt.

In another group of 5 cats, the drug in highly toxic doses was administered intramuscularly and the animals were fed daily by stomach tube with the result that the state of nutrition was better maintained. They were sacrificed in periods ranging from 1 to 7 days after the drug. The brains were fixed in 10 per cent formalin and sent to the Laboratory of Pathology (Dr. M. C. Winternitz) in New Haven for histological examination. The results are summarized in table 2. There were some histological changes in the cerebrum and cerebellum. They appeared to develop only after several days. The changes included chromatolysis of ganglion cells of Ammon's horn, diminution in the number of ganglion cells in the cerebral cortex, and perivascular lymphocytic infiltration. The changes were inconstant.

Cats—blood. The blood was studied for effects of NDR-293 on the hemoglobin, the cellular elements, the hematocrit, non-protein nitrogen, creatinine, CO_2-combining-power and prothrombin content. The results in the 8 animals are summarized in table 3. One animal was used for control purposes. In three, the study is incomplete because they represent incidental observations on the blood made during the course of other experiments. In the remaining four animals, two control samples of blood were studied before the drug and several samples taken at intervals of from 3 to 7 days after the drug. In all cases the animals were in a state of severe poisoning after the drug at the time the samples were taken. The doses of NDR-293 varied from 0.07 to 0.28 mM by intravenous injection.

There was a fairly consistent fall of the hematocrit. There was an occasional

reduction in the hemoglobin and red blood cell count. The number of white blood cells was extremely irregular. The relationships between the hematocrit, hemoglobin and red blood cell content were so irregular that one cannot detect the underlying factor at work in the changes of the cellular elements of the blood.

The non-protein nitrogen varied considerably but an unmistakable azotemia with a non-protein nitrogen of 180 mg. per 100 cc. developed in 2 cases. No significant change in creatinine occurred. The CO_2-combining-power was also not significantly altered. The prothrombin content of the blood fell in 2 cases.

TABLE 2

Brain pathology in cats poisoned by intramuscular NDR-293

CAT. NO.	DOSE OF NDR-293	SYMPTOMS OF POISONING	INTERVAL FROM FIRST DOSE TO SECTION OF BRAIN	HISTOLOGY
	mM/Kg.		*days*	
320	0.28	Typical, marked	2	Cerebrum, cerebellum, normal.
321	0.28	Typical, marked	1	Cerebral cortex, basal ganglia, cornu ammonis formations, cerebellum, pons, normal.
322	0.28 0.34 (3 days later)	Typical, marked	6	Cerebral cortical laminae, cerebellar folia, pons, normal. Ammon's horn normal except for a few chromatolytic ganglion cells.
323	0.28	Moderate	4	Cerebral cortex, basal ganglia, pons, cerebellar cortex, normal. Ischemic change in some neurons of Ammon's horn (a few cells have disappeared and loss masked by increase in astrocytes).
324	0.28	Moderate	5	Cerebellum and pons normal. In cortex number of ganglion cells absent in temporal lobe, no vascular proliferation and feeble gliosis at this site. Ammon's horn normal. In basal ganglia lymphocytic collars on some vessels, but neurons normal.

It appears that the cellular elements of the blood are not consistently altered in chronic poisoning by NDR-293, that renal damage as shown by azotemia and liver damage as shown by diminished prothrombin developed occasionally.

Dogs. The general systemic effects of NDR-293 in dogs were studied after intravenous injection and cutaneous application.

Each of 4 dogs received intravenous injections of doses ranging from 0.035 to 0.09 mM. The effects were qualitatively the same as those in cats both in regard to the immediate reaction and the prolonged poisoning. The relative sensitive-

TABLE 3

Effect of NDR-293 on the blood of cats

CAT NO	NDR 293 (vein)	DAY OF POISONING	BLOOD TESTS: Hematocrit	Hemoglobin	R B C (× 10⁶)	W B C (× 10³)	NPN	Creatinine	CO_2 combining power	Prothrombin (dilution at 15 sec.)
	mM/kg		%	Gm.			mg %	mg. %	vols. %	
181	0.21	8	39	11.8	7.0	1.95				
185	0.28	5 8	 32	 11.3	 9.0	 12 2	71.0		43.0	
186	0.07	5					53.0		41.8	
1-K*	0.11		B 30, 30 A 25	B 10.1, 10.1 A 9.8	B 7.0, 6.9 A 6.8	B 20.0, 21.0 A 19.1	B 38.5, 42.5 A 41.2	B 1.1, 1.0 A 1.0	B 41.5, 44.9 A 49.0	B 1:26, 1:25 A 1:19
2-K	0.11		B 42, 43 A 38, 39, 41, 36	B 11.5, 13.0 A 11.6, 16.0, 18.2, 15.8	B 9.3, 9.2 A 9.4, 9.7, 10.8, 9.8	B 15.9, 18.2 A 13.4, 12.2, 19.0, 18.9	B 46.5, 44.0 A 58.0, 80.0, 73.0, 33.5	B 1.5, 1.25 A 1.3, 1.25, 1.6, 0.8	B 43.8, 52.5 A 48.6, 42.5, 43.0, 41.6	B 1:21, 1:20 A 1:18, 1:6,** 1:6, 1:11
3-K*	0.11		B 36, 33 A 30, 26, 30, 22, 22, 21, 22	B 11.8, 10.9 A 9 8, 10.0, 10 5, 8.5, 9.0, 9.4, 8.8	B 6 8, 7.0 A 6.7, 4.8, 5.7, 5.6, 5.2, 5.1, 5.0	B 11.9, 22.4 A 13.6, 15.6, 14.6, 13.5, 21.5, 16.7, 15.2	B 53.0, 48.0 A 45.3, 46.0, 50.0, 53.0, 53.0, 38 0, 42 0	B 1.25, 1.0 A 1.1, 1.1, 1.3, 1.3, 0.9, 0.75, 0.8	B 31.5, 48.9 A 44.2, 42.0, 52.7, 48.6, 48.3, 48.0, 43.8	B 1:21, 1:21 A 1:21, 1:13, 1:13, 1:17, 1:16, 1:16, 1:10
1-K	0.11		B 41, 42 A 42, 38, 33 29	B 14.0, 12.5 A 12.8, 12.5, 11.8, 12.6	B 7.9, 7.3 A 7.7, 8.5, 8.9, 7.8	B 13.4, 16.7 A 15.3, 9.2, 57.7, 43.5	B 46.5, 45.0 A 64.7, 70.0, 71.0, 180.0	B 1.1, 1.25 A 1.5, 1.5, 1.75, 7.3	B 47.8, 45.0 A 43.2, 51.5, 46.0, 53.8	B 1:23, 1:20 A 1:21, 1:18, 1:18, 1:18
5-K	Control		38, 39, 36, 38, 39, 40	14.2, 11.2, 13.9, 11.2, 12 7, 14.2	8.6, 8.9, 7.9, 8.2, 7.8, 8.4	10.4, 8.3, 8.9, 9.2, 12.2, 7.9	49.0, 46 0, 39.0, 59.0, 60.0, 61.0	1.3, 1.75, 1.25, 1.3, 1.5, 1.5	43.0, 49.5, 48.4, 49.0, 51.0, 54.0	1:22, 1:21, 1:20, 1:18, 1:19, 1:18

* Fed daily by stomach tube.

** Dilution at 45 seconds.

B (Controls, one 2 days before the drug and the other on the day the drug was given).

A (First sample about 48 hours after the drug; subsequent ones at intervals of 3, 4 and 7 days).

ness of the dog to acute and chronic poisoning is not the same as that of the cat. The dog is more sensitive to the acute effects and the cat more sensitive to the chronic effects. The results indicate that the dog is about five times as sensitive as the cat to acute effects. Thus, one dog died from 0.09 mM and very severe symptoms regularly resulted from 0.05 mM, whereas cats survived the acute effects of 0.28 mM. The dog recovered quickly, usually almost completely within a few hours, and showed relatively little chronic poisoning which was the outstanding phenomenon in cats. The failure to observe more severe chronic poisoning in the dog may be due to the fact that it is not possible to give large enough doses without causing death from the acute action. The difference may also be partly due to the more rapid elimination of the compound in the dog.

The results of cutaneous application were studied in 5 dogs after doses of 0.14 to 1.04 mM. As in the case of cats, slight local irritation was produced and there was some absorption as shown by systemic effects. The systemic poisoning

TABLE 4

Effect of NDR-293 in monkey (Macacus Rhesus)

DOSE	ROUTE	ONSET OF EFFECTS	DURATION OF POISONING	REMARKS
mM/Kg.				
0.07	Vein	Immediate	5 hours Recovered	Respiration ceased, artificial respiration, salivation, convulsive jerking, diarrhea, incessant movements.
0.41 (16 days later)	Muscle	30 min.	<20 hours Recovered	Slight depression, hoarse breathing, weakness, unable to stand, constricted pupils, blinking, vomiting.
0.69 (14 days later)	Muscle	5 min.	20 hours Recovered	Crouching, depressed, tremors, rigid, expiratory grunt, prostate, does not resist handling.

after even very large doses was very slight with recovery within a few hours. The smallest dose used which produced some systemic effects was 0.38 mM, but there were marked individual variations, and a dose twice as large produced no effect.

Monkey. The effects of NDR-293 were studied in one monkey. The results are summarized in table 4. A dose of 0.07 mM intravenously produced immediate effects which nearly proved fatal. There was no tendency to chronic effects. Recovery was complete in 5 hours. Similar symptoms, but less pronounced, were produced by a dose 10 times as large by intramuscular injection. They lasted about 20 hours. The results in this experiment show that the monkey resembles the dog rather than the cat with respect to the high susceptibility to the acute effects and low susceptibility to chronic effects.

Rabbit. The effects of NDR-293 were observed in each of 2 rabbits after an intramuscular dose of 0.069 and 0.69 mM respectively. Signs of poisoning ap-

peared within about one and a half hours, rapid respiration, salivation, anorexia, abnormal head movements, nystagmus, and convulsions. Recovery was practically complete within 24 hours although nystagmus persisted for more than 5 days. The animals were sacrificed at the end of 5 days. The site of the injection in the muscle was necrotic. There was damage of the cerebral and cerebellar cortical cells.

2,3-DIMERCAPTOPROPYL ACETATE (NDR-230). *Cats—general effects—intravenous injection.* This compound was injected intravenously in the form of a 10 per cent solution in propylene glycol in each of 17 cats. The doses varied from 0.025 to 0.05 cc. The signs of poisoning included conjunctivitis with edema of the conjunctiva, blepharospasm, lacrimation, salivation, ataxia, prostration, impaired response to painful stimuli, respiratory stimulation and myoclonic convulsions. Symptoms appeared almost immediately. The initial effect in some cases was intense prostration from which the animal seemed to recover within about a minute. This may possibly be due to the immediate drop in the blood pressure which will be discussed later. The eye-signs listed above, followed within the next few minutes as well as the other symptoms of poisoning which increased in intensity until myoclonic convulsions appeared in from 7 to 63 minutes. In the fatal cases, following the doses used, animals died in from 21 to 156 minutes (average 90 minutes). In the non-fatal cases the course was also brief, recovery being apparently complete in most cases within less than 24 hours.

The LD 50 by intravenous injection is 0.036 cc. In this small series of experiments, the smallest dose which proved fatal was 0.03 cc. and the largest dose which was survived was 0.04 cc.

Cats—general effects—intramuscular injection. Observations were made on 10 cats after doses of from 0.04 to 0.75 cc. The substance was administered, as in the case of the intravenous doses, in the form of a 10 per cent solution in propylene glycol. It produced severe local irritation, as seen by the fact that immediately after the injection the cats cried and howled as if in pain. Evidence of local irritant action was confirmed at post-mortem in 2 cases.

Absorption was fairly prompt, symptoms appearing in from 11 to 46 minutes. The symptoms were substantially similar to those after the intravenous doses. The compound was approximately 30 per cent less effective by the intramuscular route. However, the toxicity was also more irregular, one animal succumbing to a dose of 0.04 cc. in a manner similar to that after such a dose given by intravenous injection, while another failed to show any effects from a dose nearly twice as large, 0.075 cc. As in the case of intravenous injections the course of the poisoning was fairly brief, terminating in death within a few hours, or in complete recovery in less than 24 hours.

Cats—general effects—cutaneous application. The effect of cutaneous application was studied in 14 cats. The drug was applied undiluted to the skin of the abdomen or thorax or both, the site having been prepared by close clipping of the hair, washing and drying. The doses varied from 0.04 to 0.2 cc. The areas covered varied from 28 to 55 square centimeters.

A strong local action at the site of application was produced. It usually ap-

peared in less than 10 minutes. Its intensity varied from animal to animal and to some extent with the dose. It consisted of erythema, edema, and later ulceration. The redness and swelling usually lasted from 3 to 4 days and the ulceration for more than 20 days, in one case longer than 85 days.

There was some absorption of the material directly applied to the skin, although the amount was very small as indicated by the slight systemic effects. With one exception the systemic effects were confined to those on the eye and the salivary gland, mainly lacrimation, blinking and salivation. These appeared in from 24 to 94 minutes after the application. They lasted a few hours; they were, with one exception, over in less than 24 hours. The feeble action by cutaneous application may in part be due to rapid elimination.

The effects of the cutaneous application in one dog were similar to those in cats, but less marked.

Cats—cardiovascular dynamics. Observations were made in 10 cats during "Dial" anesthesia. NDR-230 was administered intravenously in a 10 per cent

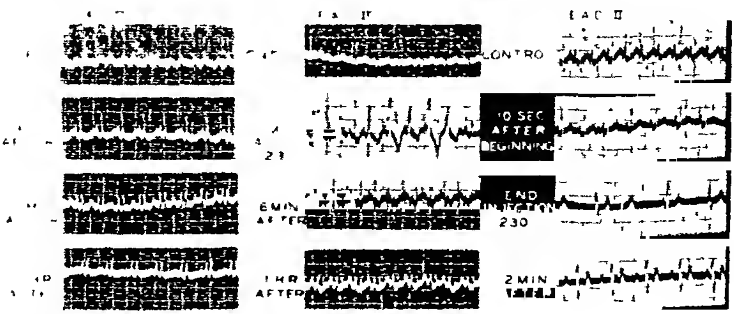


FIG 6

solution in propylene glycol. The doses ranged from 0.02 to 0.075 cc. The systemic arterial pressure fell sharply, often to shock levels, immediately after the injection, and very promptly returned to a level slightly below, or somewhat above the control value. It was then well maintained for several hours. In the case of fatal doses, after being maintained for an hour or two it began to fall off steadily, again reaching shock levels in a period of about a half hour. There was an increase in the peripheral arterial resistance as seen in the perfusion of the intact cat's leg with the Bartlett technic. A slight temporary increase in portal vein pressure was observed in one case, in another a slight temporary increase followed by a decrease of femoral vein pressure, and a moderate increase in cervical lymph flow in a third case.

In all of 3 cats in which electrocardiograms were made, changes were observed (fig. 6). The voltage of the P-waves increased and of the R-waves decreased. There was marked temporary slowing of the sinus rhythm, there were ventricular

premature contractions and A-V dissociation. The latter changes followed the larger doses. In one animal marked electrocardiographic changes were produced, but the respiration ceased during the injection and the heart continued to beat for several minutes.

The pattern of the effects on the circulation resembles more closely that of NDR-293 than that of BAL. In the range of doses used in these experiments death may be due to a combination of respiratory and circulatory failure. It is not primarily cardiac.

Cats—pathological changes. The results of post-mortem examination were studied in 27 animals. The outstanding changes were observed in the heart. The most common finding was a pericardial effusion, which was sometimes clear and straw colored, but usually sero-sanguinous. From 3 to 10 cc. was found in the pericardial sac. It formed fairly rapidly and was present in all cases which died in from 58 to 156 minutes, while those which survived similar intravenous doses and were examined 1 to 10 days later failed to show effusion or any changes which would indicate that effusion had been present and had been absorbed. It was also not dependent on poisoning severe enough to cause death, since in the fatal cases after intramuscular injection there was no effusion. It seems probable that the production of a pericardial effusion depends on a high concentration of the drug in the blood stream.

Subpericardial hemorrhages were observed in 3 cases. Subendocardial hemorrhages of varying degree were commonly seen. However, these are frequently found in normal cats and their significance remains uncertain. Hemoglobinuria was observed in 3 cats after intravenous doses.

In the case of intramuscular injection evidence of local irritation was found. In one animal which died in less than 3 hours there was edema, in another killed 6 days after the NDR-230, there was a fibrotic nodule at the site of injection. The pathological changes in the skin after cutaneous application have already been discussed.

Propanedithiol—1,3 (NDR-132). Each of 6 cats received intravenous doses of from 0.02 to 0.03 cc. of this substance in the form of a 10 per cent solution in propylene glycol. The effects resembled those of NDR-230 and NDR-293. There were crying, salivation, blepharospasm, tremors, hyperexcitability and convulsions. Symptoms of poisoning were distinct in from 4 to 12 minutes after the injection. The course of the poisoning was brief with complete recovery in 4 to 12 hours. After fatal doses, death occurred in from 26 to 37 minutes. The calculated LD 50 dose for the 6 cats was 0.026 cc.

The gross examination of the viscera post-mortem revealed no significant changes with the exception of those observed in the lungs which showed hemorrhages and edema.

1,3-Dithioglycerol (NDR-397). Each of 16 cats received intravenous doses of this compound in doses ranging from 0.005 to 0.03 cc. in a 1 per cent solution in physiological salt solution. Effects appeared immediately and resembled those of NDR-230. There was crying, excitement, signs of apprehension, tremors, ataxia, hyperexcitability, rapid respiration, pilomotor stimulation,

and in the course of from 2 to 22 minutes myoclonic convulsions. Salivation was a frequent symptom, but unlike BAL, NDR-230 or NDR-293, there were no signs of conjunctival irritation. Death occurred in from 5 to 55 minutes after doses of 0.0065 cc. or more. The larger the dose the more rapid the course of the fatal poisoning. Five animals survived doses of 0.005 or 0.0065 cc., although they developed severe symptoms of poisoning, one with convulsions. Like BAL and NDR-230, but unlike NDR-293, the course of the poisoning was brief, recovery being complete after these doses in from 6 to 15 hours. The LD 50 dose calculated from these data was 0.0061 cc. or about $\frac{1}{5}$ that for BAL.

Gross examination of the viscera post-mortem showed no significant abnormalities with the exception of the lungs. They were dark, red and engorged with blood. The blood escaped freely when the organ was sectioned, the lung then returning to its normal pale appearance. There was no pulmonary edema.

2,3-Dimercaptopropyl butyl ether (NDR-420). Each of four cats received an intravenous injection of this substance in the form of a 1 per cent solution in propylene glycol, the doses varying from 0.042 to 0.096 mM. The effects appeared promptly and were well developed in from 3 to 4 minutes. The signs of poisoning included those of conjunctival irritation, edema, blepharospasm, salivation, profound depression, ataxia, marked pupillary dilatation, hyperexcitability, myoclonic convulsions and muscular rigidity resembling that of catatonia. When the animal was put into an awkward position it was inclined to maintain it for some time. Two of the 4 doses proved fatal in 72 and 125 minutes. In one the poisoning persisted and the animal was killed during a moribund state on the third day. The remaining animal continued to show ataxia for 5 days although the other symptoms had subsided, and was found dead on the sixth day.

The gross examination of the viscera post-mortem showed no significant changes. The urine in two of the animals was red, and although it showed no red cells microscopically, the benzidine test was strongly positive, indicating hemoglobinuria.

Bis-S (Acetamidomethyl) ether of BAL (NDR-406). This compound was administered intravenously to each of 12 cats in doses ranging from 0.057 to 0.57 mM. The LD 50 calculated from the data was 0.42 mM. It caused symptoms similar to those of BAL, namely, blepharospasm, lacrimation, salivation, conjunctival edema, moderate depression and ataxia. In most of those animals which received the larger doses, and in all of those that died there was a period of violent myoclonic convulsions. Death occurred in an average period of 72 minutes, which is sooner than is the case with comparable doses of BAL. At post-mortem, most of them showed clear pericardial effusions. This also applied to several sacrificed after apparent recovery. There were no other consistent findings.

Bis-S (N-ethylacetamidomethyl) ether of BAL (NDR-407). This compound was administered intravenously to each of 14 cats in doses of from 0.28 to 0.57 mM. The calculated LD 50 was 0.52 mM. The effects were similar to those of NDR-406. All 5 cats which died had clear pericardial effusions. Con-

vulsions and death were somewhat delayed, the average time to death being 219 minutes.

Mono-S (acetamidomethyl) ether of BAL (NDR-475). This compound was administered to each of 19 cats in doses ranging from 0.09 to 1.10 mM. The calculated LD 50 dose was 0.30 mM. The average time to death in 12 animals was 77 minutes. Symptoms were in all respects similar to those of NDR-406 and NDR-407, with rather marked eye signs, but convulsions and ataxia were less pronounced. Ten of the 12 animals which died had clear pericardial effusions, but no other pathological changes.

Hexanedithiol-1,6 (NDR-139). This compound was administered by intravenous injection to each of 14 cats in doses varying from 0.02 to 0.2 cc. Symptoms appeared almost immediately, weakness, lassitude, dilatation of the pupils with sluggish response to light, and ataxia. Partial recovery occurred within a few hours, there remaining slight depression, ataxia, and sluggish response to

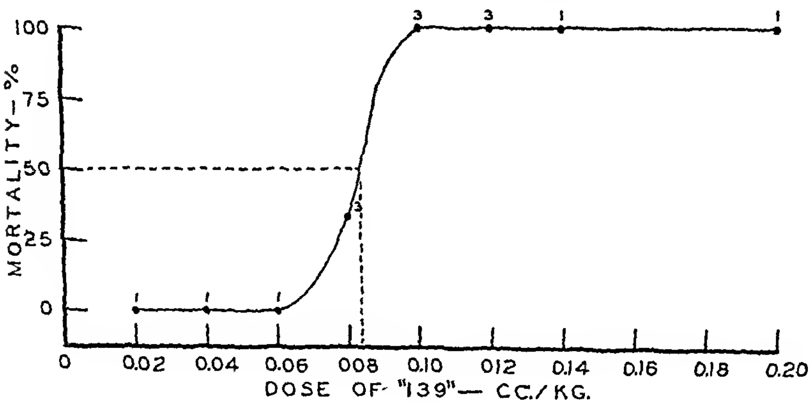


Fig. 7

pressure on the tail, although the animal appeared alert. With doses above 0.1 cc., signs of poisoning re-appeared after temporary improvement, and unlike those of BAL , progressed slowly to fatal termination in twenty-four to forty-eight hours. In the terminal stages the animals showed prostration with walking movements but no convulsions. The LD 50 was found to be 0.083 cc. or 3 to 4 times that of BAL, and the survival period was longer (fig. 7).

There were additional points in respect to which the effects differed from those of BAL. There was almost complete absence of eye symptoms, such as lacrimation and edema. Salivation was very slight or absent. In contrast to BAL, these animals lay quietly in a relaxed state while those after BAL showed unrest and hyperexcitability.

NDR-139 was tested for its effect on the isolated sartorius muscle of the frog by the technic described elsewhere (3). In a concentration of 0.001 M solution, it quickly abolished the recovery heat and induced deterioration of the mechani-

cal response. In respect to this action, it was more potent than BAL. The effect on the recovery heat was reversible; in one experiment in which a M/2000 solution completely inhibited the recovery heat, it returned to a normal value after immersion in Ringer's solution for one hour.

SUMMARY

1. This paper presents an account of pharmacologic observations on 9 dithiols, chemically related to the better known anti-lewisite BAL.

2. All of these compounds are toxic and fatal by intravenous injection in doses ranging from about 0.006 to 0.15 cc. per Kg. The range of toxicity is much narrower on a molar basis.

3. The symptoms of poisoning include the following: eye (blinking, blepharospasm, lacrimation, conjunctivitis, edema of conjunctiva); gastro-intestinal (salivation, vomiting, anorexia, diarrhea); central nervous (apprehension, depression, tremor, unrest, excitement, ataxia, convulsions, catatonia); cardiovascular (fall and rise in blood pressure, shock, arteriolar constriction, capillary paralysis with increased permeability, pulmonary edema, pericardial effusion, electrocardiographic changes, cardiac slowing and acceleration).

4. The toxicity as well as the symptoms of poisoning varied with differences in the chemical structure. For example, an exchange of the position of the —*OH* and —SH groups of NDR-133 (BAL) as in the compound NDR-397 resulted in a five-fold increase in toxicity and a disappearance of the effects on the eye. The conversion of NDR-133 (BAL) into its ethyl ether, NDR-293, left intact the circulatory changes, greatly reduced the acute toxicity, but introduced a chronic irreversible toxic action on the central nervous system never seen in case of BAL. Four of the compounds (NDR-230, -406, -407, -475) produced a pericardial effusion; from the limited experience it is not clear as to what one of the chemical changes is responsible for this action. It is noteworthy that the sulfur compound thiosinamine or allylsulphourea has been used for the production of experimental effusions (4).

5. Species differences in response to the dithiols exist; for example, NDR-293 which caused chronic changes in the central nervous system in the cat, was without this effect in the monkey and dog.

6. The duration of action is fairly brief in the case of most of the dithiols, death occurring fairly promptly, or recovery within 24 hours or less. Protracted actions over days and weeks were seen in the case of NDR-293 and -420.

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THE TOXICITIES OF COMPOUNDS RELATED TO 2,3-DIMERCAPTOPROPANOL (BAL) WITH A NOTE ON THEIR RELATIVE THERAPEUTIC EFFICIENCY[1]

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A number of thiols and derivatives related to BAL (British anti-lewisite) were submitted to this laboratory for evaluation as possible substitutes for BAL in the treatment of arsenical gas poisoning. Besides testing these compounds for their therapeutic effects in the treatment of lewisite lesions, the intramuscular toxicity, local toxicity in the eye and the toxicity by inunction into the intact skin of rabbits were obtained on most of these compounds. Some were studied thoroughly when they showed promise; others were studied only in a cursory manner when great residual damage to the animals was indicated. These compounds are listed in table 1 in the order of increasing toxicity as determined by intramuscular injection in the rat.

A. Intramuscular toxicity. Various samples of BAL and 26 compounds related to BAL were examined for systemic toxicity by intramuscular injections in rats and/or rabbits. As a standard of reference, the toxicity of BAL was carefully determined intramuscularly in rats and rabbits, intravenously in rabbits, and intraperitoneally in rats. The injections were made intravenously in the ear vein and intramuscularly in the sacrospinalis of the rabbit or in the thigh muscles of the rat. The preparations in concentrations ranging from 0.3 to 2.0 molar were usually in solution in ethylene glycol. To facilitate the inter-comparison of the compounds related to BAL, the LD50's have been expressed in table 1 in millimoles per kilogram of body weight (mM./kgm.).

When injected intramuscularly, BAL and all compounds studied showed definite toxicity to animals. Our experiments confirm the reports of Peters, Stocken and Thompson (1) and of Waters and Stock (2) that BAL produces profound toxic effects in rats. BAL has an intramuscular LD50 of about 10 mgm./kgm. (0.85 mM./kgm.) in the rat. The least toxic of the compounds studied was slightly less than half as toxic as BAL (table 1). Of the 26 compounds, 14 (Nos. 14–27) were more toxic than BAL and 4 (Nos. 24–27) were more than twice as toxic. All of these compounds studied were, within reasonable limits, of the same order of toxicity to rats as to rabbits.

B. Toxicity by inunction into rabbits' skin. The acute toxicity of BAL and 13 related compounds was investigated by application to the intact clipped skin on the back of rabbits. In the case of liquids 100% material was applied,

[1] A portion of the funds used in this investigation was supplied by a transfer, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Division of Pharmacology of the Food and Drug Administration.

TABLE 1

Toxicity of BAL and related compounds

NO.	NAME OF COMPOUND	CONCENTRATION MOLAR	INTRAMUSCULAR TOXICITY LD50		TOXICITY BY INUNCTION LD50 RABBITS	RATIO SKIN LD50/INTRAMUSCULAR LD50—RABBITS	LOCAL TOXIC EFFECTS TO SKIN OF RABBITS
			Rats	Rabbits			
			mM./kgm.	*mM./kgm*	*mM./kgm.*		
1	N-methylol-diethanolamine addition product with BAL	*		2.0	>12	>6	Moderate
2	3,4-dimercaptobutanol	2.0		1.5			
3	Bis-S (acetamidomethyl) ether of BAL	0.5	1.4	2.0	>11	>5.5	Moderate
4	2,3-dimercaptopropyl urea	1.0	1.3	0.96	6.5	6.6	None
5	Bis-S (N-ethyl acetamidomethyl) ether of BAL	0.5	1.3		>9.5		Mild
6	Dimethylamino methyl dimethyl dithiocarbamate	0.5	1.2	1.1	4.3	3.9	Severe
7	Diethanol-ammonium 1,2-dithioglycerol	0.87	1.0				
8	S (methyl glucaminomethyl) ether of 1,2-dithioglycerol	0.44	1.0				
9	N (2,3-dimercaptopropyl) carbamate	0.5		1.0	18	18	Severe
10	2,3-dimercaptopropyl ethyl ether	1.30 0.65	1.0	1.0	4.9	4.9	None
11	Methyl glucammonium 1,2-dithioglycerol	0.40	1.0				
12	S (diethanol aminomethyl) ether of 1,2-dithioglycerol	0.63	1.0				
13	BAL = 2,3-dimercaptopropanol or 1,2-dithioglycerol	1.6 1.6 0.4	0.85 0.53† 0.79‡	0.8	3.2	4.0	Mild
14	Mono-S-acetomidomethyl ether	2.0		0.8	13	16.2	Mild
15	Methyl 2,3-dimercaptopropionate	0.5	0.79		10–15	15.	None
16	1,3-dithioglycerol	1.60	0.65				
17	2,3-dimercaptopropyl methyl ether	*		0.62	3	4.7	None
18	2,3-dimercapto propionic acid	0.5	0.6		7.8		None
19	S(diethanolaminomethyl) ether of propane dithiol-1,3	0.52	0.6				
20	S(methyl glucaminomethyl) ether of trithioglycerol	0.36	0.5				
21	Propanedithiol-1,3	1.84	0.5				
22	Propanedithiol-1,2	0.92	0.5				
23	2,3-dimercaptopropyl ether	0.5	0.47	0.41	5.4	13.	None
24	Dithiobiuret	0.5	0.4	0.68	>7.4	>12.	None
25	2,3-dimercaptopropyl chloride	0.35	0.36‡				
26	Mixture of 1,2 and 1,3-dithioglycerols	1.6	0.36				
27	Propanetrithiol-1,2-3	0.5	0.3				

* Pure material used.

† Intravenous dose.

‡ Intraperitoneal dose.

while in the case of solids a saturated solution in diethylene glycol mono ethyl ether (carbitol) was used. This was done by one of two methods: (a) The material was applied under a tight rubber cuff with the animal in stocks for a period of about 17 hours as described in a previous publication from this laboratory (3), and (b) the material was rubbed into the skin with a glass rod for a period of 10 minutes and then the animal was returned to its cage. The purpose of the first method was to prevent the animals from licking the material and to prevent loss by evaporation. The approximate LD50 (2.5 mM./kgm.) of BAL determined by this method was lower than by the second method. Marked necrosis of the skin occurred in most instances. In order to simplify the test and to make possible the use of a larger number of animals, the second method was adopted. Observations over long periods showed that the animals made no attempt to lick the material. The LD50 for 100% BAL applied in this manner was 3.2 mM./kgm. Deaths occurred in a few hours and were preceded by clonic and tonic convulsions. There were no severe symptoms except in the lethal dose ranges. The local effects on the skin were much less severe than by the cuff method and showed only moderate edema, induration and redness which disappeared in the course of a few days in those animals which survived. The ratio of the LD50 by this method to that obtained by intramuscular injection in the same species gave an idea of the relative rate of absorption through the skin. Information was also obtained concerning the local toxic effects on the skin. Certain difficulties are inherent in this method of obtaining an LD50. Although it is possible to apply a volume as large as 10 cc. by prolonging the period of inunction, several of the liquids did not kill even at this dose. The majority of the solid preparations were not sufficiently soluble to incorporate a lethal dose in 10 cc. Therefore, the values given in table 1 for the LD50 by skin absorption, except for BAL and Nos. 6 ond 23, should be considered as approximate.

It is seen from table 1 that all the compounds tested by skin inunction, except No. 17, had a larger LD50 than BAL. Compounds 1, 3, 6 and 9 had moderate or severe local toxic effects to the intact skin. The ethyl ether of BAL (No. 10) caused a unique train of symptoms. About one-half hour after application the animals salivated profusely and had rapid forceful respirations. In about an hour the animals developed a tonic spasm of the extensor muscles of the neck and back with a rigid extension of the forelegs. At this time there was still profuse salivation along with clonic contractions of the jaw muscles. The spasm of neck and back muscles finally proceeded to the point where the animals fell backwards. This phase of the convulsions was followed by weak clonic twitches and then by a marked weakness of all muscles. Death occurred within 2 to 3 hours. These symptoms were noticed even in nonlethal doses, for example, 1.6 mM./kgm. The local effects were limited to a slight redness of the skin.

The derivatives of BAL tested by skin absorption consist of three types: (Nos. 6 and 24 excepted since they are of quite different chemical nature.) (a) Those with one or both SH groups modified but retaining the free OH group (Nos 1, 3, 5 and 14); (b) those retaining both free SH groups but with the first carbon radically altered (Nos. 4, 9, 15 and 18); and (c) those retaining both free SH groups but with the first carbon only slightly altered (Nos. 17 and 23).

TABLE 1

Toxicity of BAL and related compounds

NO.	NAME OF COMPOUND	CONCENTRATION MOLAR	INTRAMUSCULAR TOXICITY LD50		TOXICITY BY INUNCTION LD50 RABBITS	RATIO SKIN LD50/INTRAMUSCULAR LD50—RABBITS	LOCAL TOXIC EFFECTS TO SKIN OF RABBITS
			Rats	Rabbits			
			mM./kgm.	*mM./kgm*	*mM./kgm.*		
1	N-methylol-diethanolamine addition product with BAL	*		2.0	>12	>6	Moderate
2	3,4-dimercaptohutanol	2.0		1.5			
3	Bis-S (acetamidomethyl) ether of BAL	0.5	1.4	2.0	>11	>5.5	Moderate
4	2,3-dimercaptopropyl urea	1.0	1.3	0.96	6.5	6.6	None
5	Bis-S (N-ethyl acetamidomethyl) ether of BAL	0.5	1.3		>9.5		Mild
6	Dimethylamino methyl dimethyl dithiocarbamate	0.5	1.2	1.1	4.3	3.9	Severe
7	Diethanol-ammonium 1,2-dithioglycerol	0.87	1.0				
8	S (methyl glucaminomethyl) ether of 1,2-dithioglycerol	0.44	1.0				
9	N (2,3-dimercaptopropyl) carbamate	0.5		1.0	18	18	Severe
10	2,3-dimercaptopropyl ethyl ether	1.30 0.65	1.0	1.0	4.9	4.9	None
11	Methyl glucammonium 1,2-dithioglycerol	0.40	1.0				
12	S (diethanol aminomethyl) ether of 1,2-dithioglycerol	0.63	1.0				
13	BAL = 2,3-dimercaptopropanol or 1,2-dithioglycerol	1.6 1.6 0.4	0.85 0.53† 0.79‡	0.8	3.2	4.0	Mild
14	Mono-S-acetomidomethyl ether	2.0		0.8	13	16.2	Mild
15	Methyl 2,3-dimercaptopropionate	0.5	0.79		10–15	15.	None
16	1,3-dithioglycerol	1.60	0.65				
17	2,3-dimercaptopropyl methyl ether	*		0.62	3	4.7	None
18	2,3-dimercapto propionic acid	0.5	0.6		7.8		None
19	S(diethanolaminomethyl) ether of propane dithiol-1,3	0.52	0.6				
20	S(methyl glucaminomethyl) ether of trithioglycerol	0.36	0.5				
21	Propanedithiol-1,3	1.84	0.5				
22	Propanedithiol-1,2	0.92	0.5				
23	2,3-dimercaptopropyl ether	0.5	0.47	0.41	5.4	13.	None
24	Dithiobiuret	0.5	0.4	0.68	>7.4	>12.	None
25	2,3-dimercaptopropyl chloride	0.35	0.36‡				
26	Mixture of 1,2 and 1,3-dithioglycerols	1.6	0.36				
27	Propanetrithiol-1,2-3	0.5	0.3				

* Pure material used.

† Intravenous dose.

‡ Intraperitoneal dose.

1, 3, 6 and 9 in the table) showed moderate to severe local skin effects. In a toxic dose the ethyl ether of BAL produced a number of striking symptoms in rabbits.

3. Of 15 compounds related to BAL tested for local eye irritation, 2 (Nos. 18 and 25) were severe irritants, and 2 (Nos. 2 and 26) were more irritant in a 2.0 molar concentration than an equimolar concentration of BAL.

4. In lewisite burns of the rabbit eye only 3 compounds gave evidence of effectiveness comparable to BAL. After an exposure of 30 seconds to lewisite, number 2 in the table in a 0.5 molar solution in ethylene glycol was as effective as BAL. Compounds No. 7 and 11 were as efficient as BAL when the exposure time was 10 seconds or when they were used in aqueous solutions and the exposure was for 30 seconds.

5. In lewisite burns of the rabbit skin Nos. 1, 2, 9, 14, 16, 18, 23 and 26 were approximately as effective as BAL; Nos. 3, 4, 5, 7, 10, 15, 17, 20 and 22 were beneficial but less effective than BAL; Nos. 21 and 24 had no beneficial effect: and No. 25 was deleterious.

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Changes in structure of either type (a) or (b) have been accompanied by considerable loss of ability to penetrate the intact skin whereas changes of type (c) have had little effect on absorption.

C. LOCAL TOXICITY TO THE EYE OF THE RABBIT. By comparing their effects with BAL some of the thiols listed in table 1 were tested for local toxicity in the eye. The test materials were made up in equimolar solutions in ethylene glycol. To one eye of a rabbit weighing from 1.8 to 3 kgm. 0.1 cc. of the test solution was added and to the other the same amount of BAL or of another preparation. This procedure was repeated on other rabbits until 8 to 12 eyes were treated with each material. After 24 hours the eyes were examined for injury and a comparison was made of the effects of each material with that of the BAL standard. Of the compounds listed in table 1 and tested for local irritation in the rabbit's eye numbers 18 and 25 were found to be severe irritants. Numbers 2 and 26 were more irritating in a 2.0 molar concentration than an equimolar concentration of BAL, but in more dilute concentrations this difference was not marked. The other compounds tested (Nos. 5, 7, 8, 9, 10, 11, 12, 14, 16, 17 and 19) were about as irritating as BAL in equimolar concentrations.

D. NOTE ON THERAPEUTIC EFFICIENCY. Most of the compounds listed in table 1 were compared with BAL for their therapeutic effectiveness in the treatment of lewisite lesions of the eyes and/or of the skin of rabbits. For the lewisite lesions in the eyes only two derivatives, numbers 2 and 24 of table 1, approached BAL in effectiveness when the solvent for the compound was ethylene glycol and when the time of exposure to lewisite vapor at 22° C. was 30 seconds. The most striking way in which the majority of these compounds differed from BAL was in their failure to afford complete protection to the iris which, in nearly every case, showed damage almost equal to that found in eyes exposed to lewisite and then left untreated. When the time of exposure to lewisite was only 10 seconds, the two ammonium salts, numbers 7 and 11, appeared to be equal to the BAL standard. There was a striking increase in effectiveness of these two ammonium salts when they were made up in an aqueous solution. At a 30-second exposure to lewisite the aqueous solutions of numbers 7 and 11 were as effective for the treatment of the lesions as a similar solution of BAL.

In the treatment of skin lesions[2] produced by lewisite the compounds numbered in table 1 as 1, 2, 9, 14, 16, 18, 23 and 26 were approximately as effective as BAL. Numers 3, 4, 5, 7, 10, 15, 17, 20 and 22 were very poor therapeutic agents. Untreated lesions healed as soon or sooner than those treated with numbers 24 and 25.

SUMMARY AND CONCLUSIONS

1. The intramuscular toxicities of BAL and 26 related compounds have been studied. The LD50's varied from 0.3 to 2.0 mM./kgm.

2. Of 13 compounds tested for toxicity by inunction into intact rabbit skin, only one, the methyl ether of BAL, was as toxic as BAL. Four compounds (Nos.

[2] We are indebted to Dr. W. S. Lawrence for permission to include these data from his experiments.

STUDIES ON THE CONVULSANT ACTION OF BAL. *1. Electroencephalographic studies.*[2] Two rabbits were anaesthetized with nembutal and frontal and parietal leads were taken. The animals were injected intramuscularly with 0.3 and 0.8 mM per kg. respectively of NDR 131, a compound producing convulsive seizures similar to those caused by BAL. When the convulsions occurred, they were not preceded by increased cortical electrical activity nor was there post convulsive cortical depression. The only change in pattern was a progressive decrease in

TABLE 1

	TIME AFTER INJECTION	PULSE RATE	RESP RATE	BLOOD PRESSURE	PACKED CELL VOLUME	PLASMA pH	SERUM CO_2 CONTENT	SERUM LACTIC ACID	SERUM AMINO NITROGEN	SERUM SODIUM	SERUM CHLORIDE	BLOOD SUGAR	URINE SUGAR
				mm Hg	%		*m eq /l*	*m eq /l*	*mgm. %*	*m eq /l*	*m eq. /l*	*mgm %*	
Dog #244	Control	129	24	125	42.2	7 36	21.1						
BAL on skin	10 min	114		120	41.7	7.41	19 9						
1.8 gm /kg	1 hr. 10 m	123	24	120	44.0	7.38	14.9						
	2 hr 45 m	210	42	118	52.0	7.16	5 2						
	5 hr. 15 m	140	36	25	46 5	6 78	2 3						
Dog #287	Control	120	panting		29 6	7 35	19.3	4 8	5 4			92	0
BAL	1 hr. 10 m.	216	39		35.5	7.23	7.6	11 9				265	+
Intramuscular	2 hr. 20 m	180	57	64	37.2	7.16	4.1		10 2			360	+++
0.81 mM/kg	4 hr. 10 m	169	48	64	42 0	7 09	2 8		11.8			350	++++
	6 hr	147	65		43.8	7.05	2 2	14.8	15 6			110	++++
Dog #251	Control	108	panting	110	49.3							90	
BAL	Control	96	panting	168	48.4	7 35	19 2			140 6	101.6		
Intramuscular	23 min	180	42	150	48.1	7.38	15 4			140 6	104.8	124	
0.97 mM/kg	50 min	240	128	110	50 0	7.37	6 4			136 8	103.2		
	1 hr. 46 m	128	136	110	51.9	7.34	4 0			136.3	101.6	232	
	3 hr. 20 m.	165	36	40	53 0	7.14	3 1				105.2	48	
Dog #247	Control	87	panting	142	38 2								
BAL	12 min	240	72	80	54.4								
Intravenous	36 min	236	60	64	54.5								
0 73 mM/kg in 3% saline	53 min.	204		92	57 2	7 23	7 6						
Dog #328	Control					7.40	21 3					65	
BAL	35 min.					7.41	8 5					175	
Intramuscular	2 hr. 55 m					7.25	3.4					90	
0 81 mM/kg	4 hr. 20 m					6.94	7.4*					<20	

* Respiration ceased prior to removal of arterial sample

cortical activity beginning soon after injection. The experiments indicated that the convulsions were not of cortical origin.

2. Effect of transection of spinal cord. Section of the thoracic cord abolished convulsive movements of the hind legs of a rabbit injected with 1-3 dithioglycerol. Tonic and clonic activity of the forelegs was uninhibited.

[2] These experiments were conducted in conjunction with Dr. Leslie F. Nims of the Department of Physiology, Yale University School of Medicine.

THE TOXICOLOGICAL ACTION OF 2,3-DIMERCAPTOPROPANOL (BAL)[1]

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The assignment of screening of pilot plant samples of BAL and analagous compounds for toxicity and therapeutic efficiency was given to project OEMcmr 39. In association with these routine tests, a few experiments were performed to elucidate the toxicological action of BAL in experimental animals. The results of these experiments are reported here.

MATERIALS. Twenty-eight different preparations of BAL and 47 compounds analagous to BAL, prepared under the direction of Dr. W. A. Lazier of the E. I. DuPont de Nemours Research Laboratory, were tested for toxicity and therapeutic efficacy against Lewisite skin burns. Only three were used in the studies on toxicological action reported here. The earliest experiments were done with the first American preparation of BAL, hereafter called PP_1, a toxic sample with an LD 50 of 0.2 mM per kg. when injected intramuscularly into mice. The studies on the effects of anaesthesia, transection of the spinal cord, and the electroencephalographic changes during convulsions were done with NDR 131, 1-3 dithioglycerol; this material had convulsant action and toxicity equal to PP_1. The remaining studies were carried out using A.R.S., a standard pooled sample of 10 pilot plant preparations of BAL with an LD 50 of 0.8 mM per kg. when injected intramuscularly, subcutaneously or intraperitoneally into mice, rabbits, and rats.

SYMPTOMS OF BAL POISONING. Following administration of toxic, sublethal doses of BAL to mice, rats, guinea-pigs, rabbits, and dogs there is an initial period of apathy accompanied by lacrimation, blepharospasm and edema of the conjunctiva. Dogs salivate and frequently retch and vomit. These symptoms may regress and leave no residual findings. With large doses there are, in addition, muscle tremors which gradually increase in intensity; the animals lose coordination and soon generalized tonic and clonic convulsions occur. Only a rare animal recovers from the convulsion stage; the usual course is characterized by an increase of depth and rate of respiration, rapid thready pulse, nystagmus, coma alternating with repeated convulsions, and death.

EFFECT ON THE VASCULAR SYSTEM. An early transient rise in blood pressure is occasionally seen but animals receiving lethal amounts of BAL all exhibit preterminally signs of peripheral vascular collapse as evidenced by rapid pulse, hypotension and increase of packed cell volume (table 1).

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Yale University during the years 1942 and 1943.

rabbits given 0.33 mM per kg. of PP_1 were anaesthetized for four hours and recovered completely within fifteen hours. Unanaesthetized rabbits given a similar dose had succumbed within thirty minutes. Two rabbits given .66 mM per kg. of PP_1 were not protected by anaesthesia, but the survival period was prolonged to one and a half hours.

ALTERATIONS OF ACID-BASE EQUILIBRIUM, CARBOHYDRATE METABOLISM AND LIVER COMPOSITION. The results of determinations on five dogs following administration of lethal doses of ARS by various routes are recorded in table 1. The pH of the blood is reduced as is the plasma CO_2 content. From values for arterial plasma pH and CO_2 content the carbon dioxide tension and the bicarbonate content may be calculated by application of the Henderson Hasselbach equation. The values so obtained in four of these dogs have been plotted on triaxial coordinates (fig. 1) as described by Shock and Hastings (1) (2). The observed acidosis is attributable chiefly to accumulation of serum lactic acid; there is little loss of serum sodium in the one animal so studied. The urine remains free of acetone and diacetic acid. An early rise in blood sugar accompanied by glycosuria is followed by preterminal hypoglycemia. There is a rise of serum amino nitrogen.

TABLE 2

The effect of BAL on liver composition and blood sugar

DOG NUMBER	INTERVAL BETWEEN INTRAMUSCULAR INJECTION OF 0 81 mM BAL/kg (100 MGM /KG) AND SACRIFICE	BLOOD SUGAR WHEN SACRIFICED	LIVER GLYCOGEN	LIVER*			
				H_2O	Cl	Na	K
		mgm. %	gm. %	gm.	mM	mM	mM
Normal		60–100	Variable	270–300	10–12	10–12	32–38
332	30 min.	123	5.82	310	10.5	15.7	34.4
331	1 hr. 25 min.	180	1.03	300	15.1	19.1	25.7
333	2 hr. 10 min.	272	1.03	300	16.0	21.6	22.8
328	4 hr. 20 min.	20	0.0	367	21.5	30.0	26.4
287	6 hr. 20 min.		0.0	421	38.5	48.7	14.6

* All values expressed per 100 grams of fat free dried tissue.

Alterations of liver composition following administration of BAL to five dogs are shown in table (2).[3] Depletion of hepatic glycogen was complete when hypoglycemia occurred. There was a marked decrease of potassium and an increase in concentration of chloride and sodium. Despite these extreme changes in the liver electrolyte pattern no histological evidence of hepatic cell necrosis was found.

DISCUSSION. Convulsions produced by BAL are not the result of hypoglycemia since they occur when the blood sugar is not reduced. They are subcortical in origin as shown by electroencephalographic studies. Spinal section abolishes convulsive action in the denervated extremities indicating that con-

[3] These determinations were made by Dr. Daniel C. Darrow of the Dept. of Pediatrics, Yale University School of Medicine, by methods already published (4).

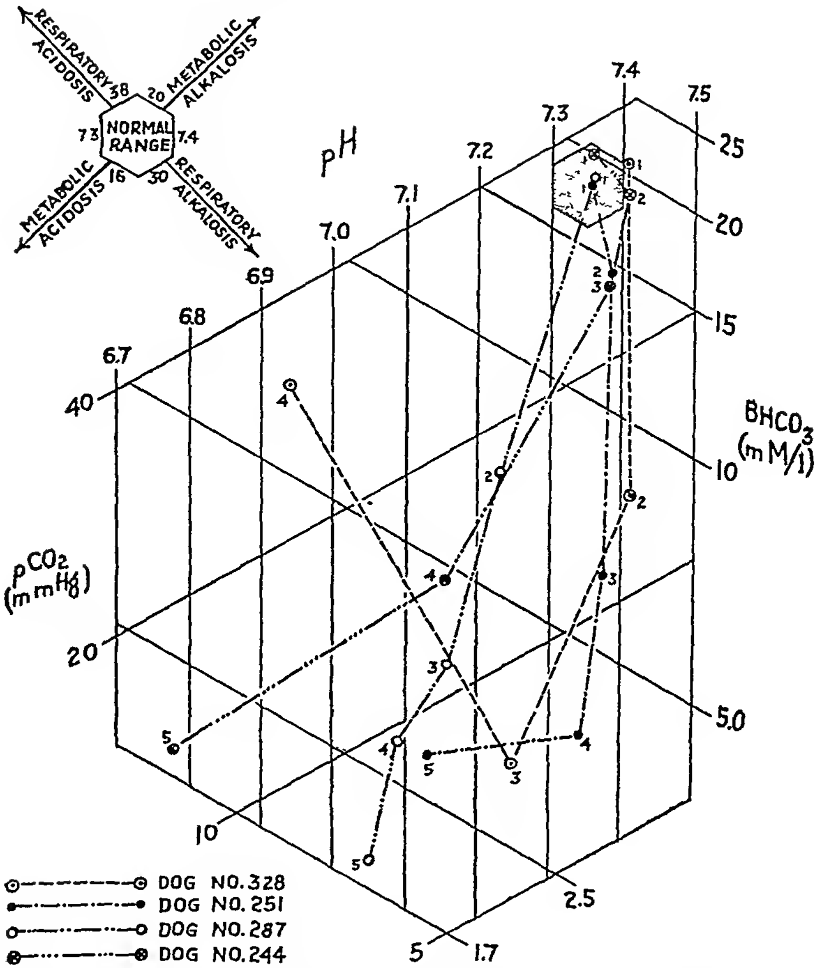

FIG. 1. Values for arterial plasma pH, of plasma CO_2 tension, and bicarbonate are plotted according to the method of Shock and Hastings (1) (2). The range of normal values (3. are indicated by the small shaded hexagon. Elapsed time between the administration of BAL and the time that the samples of arterial blood were withdrawn are charted below (For additional data on these dogs see table 1.)

	Dog #328	*Dog #251*	*Dog #287*	*Dog #244*
Sample 1	Control	Control	Control	Control
Sample 2.	35 min.	23 min.	1 hr. 10 min.	10 min.
Sample 3.	2 hr. 55 min.	50 min	2 hr. 20 min.	1 hr. 10 min.
Sample 4	4 hr. 20 min	1 hr. 45 min	4 hr. 10 min.	2 hr. 45 min.
Sample 5		3 hr. 20 min.	6 hr.	5 hr. 15 min.

3. *Effect of anaesthesia.* Rabbits were anaesthetized with intravenous sodium pentobarbital and subsequently injected intramuscularly with PP_1. Sodium pentobarbital injections were repeated as necessary to control convulsions. Two

TOXICITY OF 2,3-DIMERCAPTOPROPANOL (BAL) IN CATS[1]

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A detailed investigation of the toxicity and behavior of BAL in the cat was carried out in our laboratory. This report presents an account of these experiments.

Signs and symptoms. The effect of BAL in the normal cat was studied in detail in 33 animals after a total of 173 intravenous injections of doses varying from 0.005 to 0.03 cc. per Kg.[3] The compound was injected undiluted, in pure propylene glycol, or in saline, in the solutions usually of 1 per cent. The smallest dose which produced visible effects gave rise to signs referable to the eyes and salivary glands, blinking, lacrimation, salivation. These developed within about 10 minutes. They progressed and within half an hour there was slight edema of the conjunctiva with congestion. These symptoms subsided within 1 to 2 hours. The minimal doses produced no evidence of other systemic reactions. The foregoing symptoms were intensified and others were added after larger doses. After a dose of 0.01 cc., the blinking developed within about 5 minutes, lacrimation and drooling of saliva within about 10 minutes, and blepharospasm with edema of the conjunctiva within 20 to 30 minutes. Urination almost invariably occurred within 10 to 40 minutes. The odor of BAL was detectable in the expired air within 3 to 5 minutes. With this dose, which was twice as large, the symptoms lasted about twice as long, about 4 hours.

After still larger doses, 0.02 cc., the above symptoms appeared in greater intensity and reached a peak in about an hour. Two new symptoms were produced; marked ataxia, especially of the hind limbs; and, evidence of analgesia, so that stepping on the tail failed to elicit response. The gait was staggering, wide-based, and the incoordinated movements of the hind limbs often caused the animal to fall. There was some hyperexcitability although convulsions could not be induced. The respiratory rate was increased. Even such advanced poisoning was reversible and within 4 to 5 hours all symptoms disappeared.

Larger doses, 0.03 cc., frequently proved fatal. The latent period was shorter, symptoms appearing within 2 to 10 minutes. They progressed more rapidly, the animal being unable to stand within 15 minutes. Rapid gasping respiration developed, often with loud rhonchi and frothy bloody sputum. Myoclonic convulsions appeared in from 20 to 30 minutes; these were frequently, although not always, associated with a fatal outcome. In 7 fatal cases following this dose, the average survival time was 133 minutes.

[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

[3] All doses were given in terms of body weight, hence "per Kg." will not be repeated.

vulsions are neither of spinal nor of peripheral origin. The exact site of origin of these convulsions has not been further elucidated.

The changes in the constituents of the plasma and composition of the liver produced by BAL and reported here are similar to those previously described in hemorrhagic shock; namely, a rise in amino nitrogen concentration of serum (5), a rise in serum lactic acid (5), depletion of liver glycogen (6), and loss of hepatic potassium accompanied by a rise of hepatic sodium and chloride (7). These changes when found in states of shock have been attributed to inhibition of hepatic enzyme activity secondary to reduced blood flow through the liver and resultant anoxia (7). In BAL poisoning however the metabolic acidosis and changes in blood glucose concentration occur before there is peripheral circulatory collapse (cf. Dog #251, table 1), indicating that these metabolic disturbances result from direct inhibition by BAL of liver enzyme systems (8).

SUMMARY AND CONCLUSIONS

BAL is a toxic drug, producing lacrimation, salivation, vomiting, tremors, convulsions, coma, peripheral circulatory collapse and death in experimental animals.

The convulsions following BAL are not due to hypoglycemia and the mechanism of their production is not known. They may be alleviated by anaesthesia with sodium pentobarbital.

Toxic doses of BAL produce a metabolic acidosis as indicated by diminished blood pH and CO_2 content and increased serum lactic acid and amino nitrogen. The blood sugar rises initially but falls to hypoglycemic levels before death.

Liver glycogen content is reduced in BAL poisoning and the liver potassium is decreased with increase of the liver sodium and chloride.

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respiration up to 222 a minute, followed by slow gasping respiration, loud rhonchi, and bloody frothy cough (pulmonary edema). Instead of the protracted convulsive state of BAL, the animal developed a state of catatonia, maintaining the position into which it was forced. The eye-signs and salivation characteristic of BAL were absent.

SITE OF ACTION PRODUCING LACRIMATION AND SALIVATION. Since after intravenous doses of 0.01 cc. of BAL, the odor of the drug is detectable in the expired air, it seemed possible that the salivation (drooling) and eye-signs might be the result of a local action of BAL in the air rather than of a systemic action of BAL. Accordingly, in each of 2 cats, during local anesthesia, a tracheal cannula was inserted in such a way as to divert the exhaled air from the mouth, nose, and face. After intravenous doses of 0.01 cc. of BAL, salivation, lacrimation, and blinking appeared in the usual time, and the intensity of effects were indistinguishable from those seen in normal cats. It is clear, therefore, that the salivation and eye-signs are the result or BAL acting through the circulation.

The rôle of parasympathetic innervation was studied in each of 3 cats. On alternate days the animals received a control intravenous dose of 0.01 cc. of BAL, and a similar dose of BAL preceded by an intravenous injection of 0.1 mg. of atropine sulfate. This dose of atropine had no effect on the blinking, blepharospasm and conjunctival edema produced by the BAL, but markedly reduced the salivation and lacrimation. A larger dose of atropine, 0.5 mg. (on the following day) prevented all salivation and lacrimation, but was still without influence on the other eye symptoms of BAL. The animal was found to be normally responsive to BAL on the day after the larger dose of atropine. These experiments indicate that parasympathetic innervation is necessary for the action of BAL on the salivary and lacrimal glands. They also indicate that the conjunctival edema and blepharospasm are independent of any BAL which might be excreted in the tears.

RESPIRATION. BAL produces both stimulation and depression of respiration. There is stimulation with moderate doses and in the early phases of poisoning. Profound respiratory depression usually precedes death. In some cats the effect on respiration closely resembles that of nicotine; a short period of depression followed by acceleration; with successive injections the depression becomes progressively greater while the acceleration phase gradually disappears. It has not been determined to what extent the respiratory changes are the result of direct action on respiration and indirect action through the changes in the circulation.

CIRCULATION. The actions of BAL on the circulation are complex. Small doses produce a rise of the blood pressure while larger doses, a fall of the blood pressure to shock levels. The primary actions appear to involve a direct constrictor action on the arterioles of the limbs and a direct capillary injury. The detailed analysis of the circulatory changes are presented in another communication (2).

EFFECT ON BLOOD pH AND CO_2 COMBINING POWER. The effect of BAL on the blood pH and CO_2 combining power was tested in each of 3 cats after an intravenous injection of 0.03 or 0.04 cc. These doses proved fatal in 53 to 108

Toxicity of various samples of BAL. Results in our laboratory are in line with those of other workers (1) showing that various of the early samples of BAL differed in toxicity. The results obtained in 46 cats with 3 samples of BAL are shown in log-dose mortality curves in figure 1. The intravenous LD50 dose of the American Reference Standard BAL in cats proved to be 0.032 cc. per Kg.

Effect of injected lactic acid. The observation of Bunting, Ordway, Harrison, Durlacher, and Albrink (referred to by Waters and Stock [1])to the effect that toxic doses of BAL lead to the accumulation of lactic acid in the blood suggested the possibility that this might be responsible for the symptoms of BAL poisoning. Accordingly, the effect of lactic acid injection was tested although

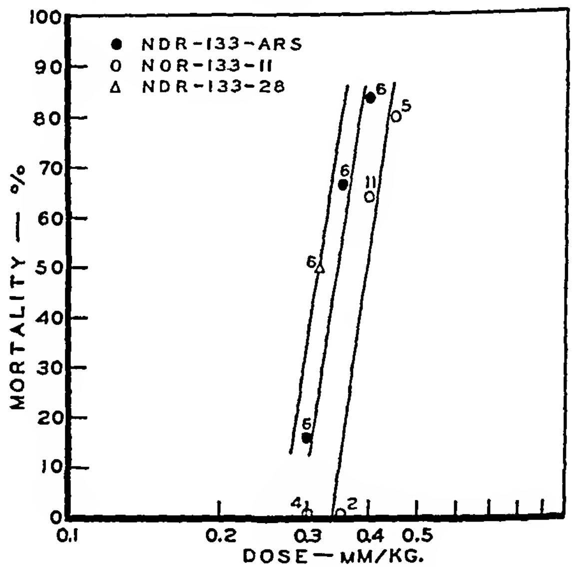

Fig. 1. Log-dose-mortality Curves for Three Specimens of BAL

Numbers at each point refer to number of animals. The mM may be converted into cc. by shifting decimal one place to the left.

there may be differences between the effect of lactic acid gradually formed after BAL and that injected at one time. Each of 3 cats received an intravenous injection of 100, 200 and 300 mg. respectively, of lactic acid in physiologic salt solution. The smallest dose produced no visible effects. The largest dose proved fatal within less than 1 minute with violent myotonic and myoclonic convulsions. The 200 mg. dose proved fatal in 40 minutes with terminal convulsive movements. The train of symptoms following this dose showed points of similarity with those of BAL but the design was sufficiently different to make it improbable that lactic acid is the cause of the symptoms of BAL. Within 30 seconds the cat collapsed, and later showed ataxia, twitching, markedly increased

counts an average increase of 38 per cent, and the non-protein nitrogen an average increase of 30 per cent (fig. 3). The cause of these changes remains unexplained. In view of the foregoing findings, it does not appear to be a matter of changes in blood concentration.

PATHOLOGY. The lungs, heart, spleen, adrenals, liver, kidneys, and intestines were examined grossly and in many instances microscopically for structural

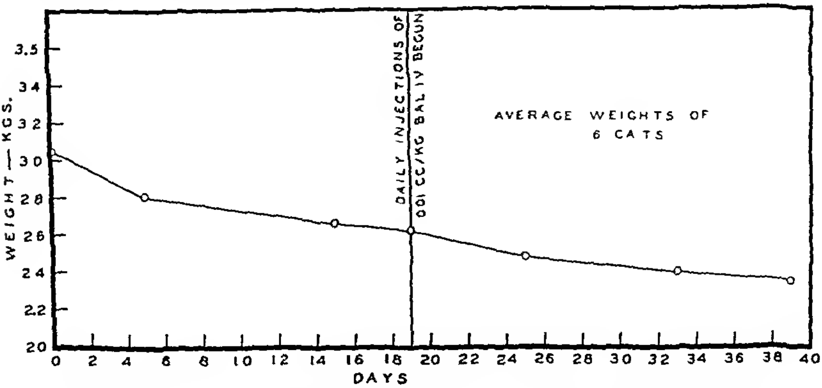

FIG. 2. EFFECT OF PROTRACTED DAILY INJECTIONS OF BAL ON WEIGHT OF CATS

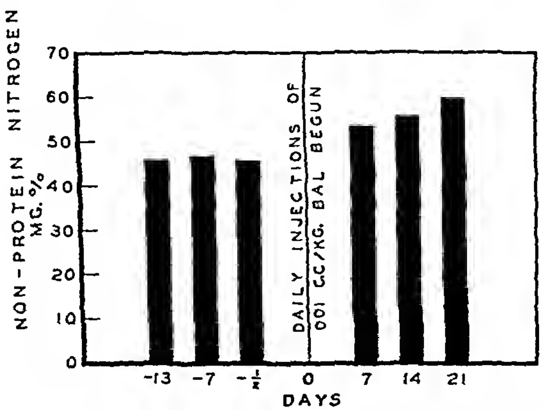

FIG. 3. EFFECT OF PROTRACTED DAILY INJECTIONS OF BAL ON BLOOD NON-PROTEIN NITROGEN OF CATS

changes resulting from single doses of BAL as well as after long periods of repeated doses. In most organs there were no conspicuous morphological changes. Isolated instances of tubular degeneration in the kidneys, and thickening of the alveolar walls in the lungs after repeated doses were observed. It is difficult to be certain of the relationship between the BAL and some of these isolated morphological abnormalities. Pulmonary edema with hemorrhages throughout

minutes. Control observations were made and again during the peak of poisoning (drooling, gasping respiration, convulsions). The pH of the blood was obtained by diluting the blood 5 times with neutral saline in which the glass electrodes of a Leeds and Northrup pH-meter had been previously immersed and over which a layer of mineral oil had been spread. In this way the blood was kept under oil, yet no oily film covered the electrodes. The CO_2 combining power was determined by the method of Van Slyke. Table 1 summarizes the results. BAL reduced the blood pH from the average control of about 7.53 to 7.15, and the CO_2 combining power from an average control of 43.1 to 25.5 volumes per cent. Similar results have been obtained by Bunting et al (referred to by Waters and Stock [1]).

EFFECT OF PROLONGED ADMINISTRATION OF BAL. This was studied in each of 6 cats which received an intravenous injection of 0.01 cc. daily for 17 injections over a period of 20 days. The cats were maintained in separate metabolism cages on a constant diet of dehydrated animal food and without restriction of water. They were weighed at weekly intervals during the control period of 15

TABLE 1

Effect of BAL on pH of whole blood and CO_2 combining power of serum

CAT. NO.	DOSE	pH		CO_2 COMBINING POWER, VOLS. PER CENT			SURVIVAL TIME
		Control	After BAL	Control	After BAL	Per cent change	
	cc./Kg.						*min.*
68	0.03	7.63	7.42 (31)†	47.9	30.3 (31)†	−36.8	91
69	0.04		6.8 (52)	38.6*	19.6* (44)	−49.4	53
70	0.03	7.43	7.20 (58)	42.8*	26.7* (58)	−37.6	108

* These samples were kept overnight in the ice-box.
† Time in minutes after the drug was given.

days and the experimental period of 20 days. Three blood specimens were studied in the control period and similarly during the period of treatment with the drug. The blood specimens were always taken 24 hours after the last injection.

Figure 2 shows the weight changes of the animals during the course of this experiment. There was a gradual decrease of weight of cats kept in small cages over a long period of time, the trend being essentially the same during the control and treatment periods.

The results showed that prolonged administration of fairly large toxic doses of BAL produces no significant sustained changes in the red blood cell count, prothrombin time, hematrocrit, CO_2 combining power, blood glucose, and creatinine. Absorption spectra of the final blood samples were negative for methemoglobin. The urine was negative for glucose and showed only the occasional traces of albumin found in the control periods.

However, the chronic exposure to BAL was not without some effect. The hemoglobin showed an average reduction of 15 per cent, the white blood cell

animals anesthetized with "Dial" in which BAL in doses of 0.15 to 0.30 cc. was applied to the whole ventral surface of the cat, only borderline effects were produced as measured by the blood pressure, peripheral resistance and lymph production.

The BAL applied directly to the skin caused local edema with injury of the capillaries as seen from the fact that an injection of 5 mg. of the blue dye "T-1824" given intravenously within a few minutes after the application of the BAL stained the area a deep blue. Although edema developed rapidly, there seemed to be no indication of sensory disturbances since the animal seemed to make no attempt to lick the area. It may be noted that the edematous area appeared to interfere with further absorption of the BAL since repeated applications to such an area failed to intensify the symptoms.

PERSISTENCE OF ACTION. Attention has already been called to the fact that, within limits, poisoning by BAL seems to be completely reversible, that after about two-thirds of an average lethal dose (0.02 cc.) injected intravenously, which produces fairly advanced poisoning, effects, as judged by the general behavior of the animal, disappear within about 5 hours. The elimination of BAL is fairly rapid. Each of 6 cats which received a daily intravenous dose of about one-third of an average lethal dose (0.01 cc.) for 17 doses failed to show significant cumulation and all survived. However, the cat is unable to recover completely in 24 hours from twice that dose (0.02 cc.), and its repetition daily proved fatal in about a week. Additional information was obtained in the case of each of 2 animals which received an intravenous dose of 0.01 cc. every hour. One died after 3 and the other after 5 doses, indicating that only a small part of the effect of such a dose (about one-third of the LD50) disappears in one hour. While in the majority of instances, recovery or death from BAL is fairly prompt, there are conditions of poisoning under which the fatal course is more protracted, as was seen in the case of each of 2 animals which received an intravenous injection of 0.005 cc. of BAL at half-hour intervals for 6 and 7 doses respectively; one died 48 hours later, and the other in 6 days. In view of the more common experience indicating rapid elimination, these 2 cases suggest the persistence of an irreversible injury independent of the elimination of the drug.

SUMMARY AND CONCLUSIONS

1. The earliest external signs of BAL poisoning in cats are blinking, blepharospasm, lacrimation, conjunctival edema, and salivation. Larger doses also produce ataxia, urination, and respiratory stimulation. After fatal doses there is added, respiratory depression, pulmonary edema, and convulsions.
2. The intravenous LD50 dose of the American Reference Standard BAL in cats is 0.032 cc. per Kg.
3. The odor of BAL appears in the expired air after intravenous injection.
4. Evidence is adduced indicating that the high blood lactic acid resulting from BAL is probably not the cause of the symptoms, that the eye-signs and salivation are produced by the BAL in the circulation and not that in the expired air, that

the lungs was a fairly common finding after severe poisoning; this was confirmed both grossly and microscopically. It was more pronounced when undiluted BAL was injected than after solutions in saline.

Absorption from skin. In view of the fact that the use of BAL as an antidote to lewisite poisoning requires its direct application to the skin, experiments were carried out to test its absorption from the skin. Waters and Stock (1) referred to various observations of Sulzberger, Baer, and Kanof, and those of Wilson and Talbot, which showed that absorption from the skin in man is very poor since an inunction of 1 cc of undiluted BAL or of 2 Gm. in a jelly base produced no systemic effects. The larger dose is about 10 times as large as the dose which was found to produce symptoms in man after intramuscular injection in another study (3).

TABLE 2

Symptoms resulting from the cutaneous application of BAL in unanesthetized cats

CAT NO	WEIGHT	DOSES OF BAL		ESTIMATED AREA	SYMPTOMS*
		Total	Per kilo		
	kg	cc	cc	cm²	
216	3 67	0 179	0 049	25	Mild
216	3 67	0 546	0 149	300	Severe†
217	4 05	0 119	0 029	25	Slight
218	2 57	0 179	0 069	25	Mild
219	3 73	0 407	0 109	25	Slight
220	2 76	0 407	0 148	45	?
221	3 46	0 446	0 129	25	Mild

* In indicating severity of symptoms "slight" represents barely perceptible effects, such as minimal salivation and blepharospasm, "mild" indicates salivation, blepharospasm, lacrimation and slight ataxia, "severe" the foregoing symptoms to a greater degree accompanied by convulsions

† Died 5 hours after application following a period of severe convulsions

BAL was applied to the surface of the skin in each of 6 cats. The hair was closely clipped over the ventral abdominal and thoracic regions, care being taken to avoid abrasion. With the cat tied on the animal board the undiluted BAL was applied and uniformly distributed by means of a glass rod over an area of about 25 cm². in most cases, and in doses of from 1 to 5 times the intravenous LD50. The animal was released after an hour. The data are summarized in table 2. It may be noted that BAL is absorbed when applied directly to the skin, but that only minimal effects result from even very large doses applied to an area of 25 cm². The effects were usually less than those produced by an intravenous injection of 0.005 cc. per Kg., indicating that the effective absorption is of the order of 3 per cent or less under these conditions. The size of the skin area to which the BAL is applied is of great importance, since a dose which produced only a slight effect when applied to 45 cm²., proved fatal when applied to an area about 7 times as great. There is, however, great variation, and in 4 additional

animals anesthetized with "Dial" in which BAL in doses of 0.15 to 0.30 cc. was applied to the whole ventral surface of the cat, only borderline effects were produced as measured by the blood pressure, peripheral resistance and lymph production.

The BAL applied directly to the skin caused local edema with injury of the capillaries as seen from the fact that an injection of 5 mg. of the blue dye "T-1824" given intravenously within a few minutes after the application of the BAL stained the area a deep blue. Although edema developed rapidly, there seemed to be no indication of sensory disturbances since the animal seemed to make no attempt to lick the area. It may be noted that the edematous area appeared to interfere with further absorption of the BAL since repeated applications to such an area failed to intensify the symptoms.

Persistence of action. Attention has already been called to the fact that, within limits, poisoning by BAL seems to be completely reversible, that after about two-thirds of an average lethal dose (0.02 cc.) injected intravenously, which produces fairly advanced poisoning, effects, as judged by the general behavior of the animal, disappear within about 5 hours. The elimination of BAL is fairly rapid. Each of 6 cats which received a daily intravenous dose of about one-third of an average lethal dose (0.01 cc.) for 17 doses failed to show significant cumulation and all survived. However, the cat is unable to recover completely in 24 hours from twice that dose (0.02 cc.), and its repetition daily proved fatal in about a week. Additional information was obtained in the case of each of 2 animals which received an intravenous dose of 0.01 cc. every hour. One died after 3 and the other after 5 doses, indicating that only a small part of the effect of such a dose (about one-third of the LD50) disappears in one hour. While in the majority of instances, recovery or death from BAL is fairly prompt, there are conditions of poisoning under which the fatal course is more protracted, as was seen in the case of each of 2 animals which received an intravenous injection of 0.005 cc. of BAL at half-hour intervals for 6 and 7 doses respectively; one died 48 hours later, and the other in 6 days. In view of the more common experience indicating rapid elimination, these 2 cases suggest the persistence of an irreversible injury independent of the elimination of the drug.

SUMMARY AND CONCLUSIONS

1. The earliest external signs of BAL poisoning in cats are blinking, blepharospasm, lacrimation, conjunctival edema, and salivation. Larger doses also produce ataxia, urination, and respiratory stimulation. After fatal doses there is added, respiratory depression, pulmonary edema, and convulsions.
2. The intravenous LD50 dose of the American Reference Standard BAL in cats is 0.032 cc. per Kg.
3. The odor of BAL appears in the expired air after intravenous injection.
4. Evidence is adduced indicating that the high blood lactic acid resulting from BAL is probably not the cause of the symptoms, that the eye-signs and salivation are produced by the BAL in the circulation and not that in the expired air, that

the salivation and lacrimation requires the mediation of the parasympathetic nervous system, and that the conjunctival edema and blepharospasm are independent of BAL which might be excreted in the tears.

5. An intravenous toxic dose of BAL lowers the blood pH and CO_2 combining power.

6. Prolonged daily administration of about one-third of the LD50 dose of BAL is without effect on the red blood cell count, prothrombin time, hematocrit, CO_2 combining power, blood glucose, and creatinine. Such doses do not produce methemoglobin. They give rise to no changes in the urine. However, they reduce the blood hemoglobin, increase the white cell count, and raise the blood non-protein nitrogen.

7. The only unequivocal morphological effect of BAL injected intravenously is edema and petechial hemorrhages in the lungs.

8. BAL is absorbed when applied directly to the intact skin, and although a fatal dose may be thus absorbed, the amount absorbed is usually quite small, variable, and depends on the size of the area of skin to which it is applied. The effective absorption is usually less than 3 per cent of the dose.

9. The toxic actions of BAL are usually reversible. BAL is rapidly eliminated; animals severely poisoned by about two-thirds of an LD50 dose may be free of symptoms within about 5 hours. Animals usually succumb to fatal doses within a few hours although occasionally a fatal course may be protracted for several days.

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THE CARDIO-VASCULAR ACTIONS OF 2,3-DIMERCAPTOPOPANOL (BAL)[1]

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Early in fall of 1942 BAL was delivered to this laboratory for pharmacological investigation. BAL possesses a number of pharmacological actions of varying degrees of importance. The general response of animals to BAL and the toxicity data will be reported separately (1). It soon became apparent that the most important systemic actions of BAL were exerted on the central nervous and the cardiovascular systems. Stimulation of the nervous system can be suppressed by barbiturates although there is but little protection against death. During routine examination of anesthetized cats it was observed that the arterial pressure regularly fell to shock levels following the injection of BAL. No evidence was elicited that death was the result of any action on the nervous system. Because of the presence of such a pronounced effect upon the circulatory system the detailed investigation described in the present communication was undertaken.

SYSTEMIC ARTERIAL PRESSURE. The effect of intravenous injections of BAL on the systemic arterial blood pressure was determined in 45 cats, 5 under local anesthesia and 40 under anesthesia induced by various barbiturates,[3] and occasionally ether. Blood pressure was recorded from a carotid artery with a Hurthle manometer on a smoked drum. Chlorazol pink (2) was usually employed as an anti-coagulant in these and other experiments. BAL was injected as a one per cent solution in physiological saline.

The results in anesthetized and unanesthetized animals were essentially the same. Single doses of BAL sufficient to cause a change in the blood pressure invariably depressed it. The usual result was an abrupt fall of the pressure immediately after the injection, with a rapid return toward, but not usually to, the control level. A secondary decline in pressure then set in which in the course of an hour reduced the pressure to 50 mm. of Hg or less. This lasted for another hour more or less before recovery or death supervened. In general, the rate and degree of depression of the blood pressure were related to the size of the dose. Approximately forty per cent of the LD50 (0.10 mM/Kgm. of this sample of

[1] The work described in this paper was done under contract, recommended by the committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

[3] Dial-Ciba (diallybarbituric acid, ethyl urethane, urethane solution) was used as the anesthetic in the majority of all experiments. Sodium amytal, sodium barbital and sodium pentobarbital were occasionally employed.

BAL) usually produced a moderate fall of pressure of the order of 20 mm. of Hg which persisted for 10 minutes or longer. A second similar dose reduced the pressure rather gradually to lower levels. The effect of two such doses at intervals of 10 or 15 minutes was essentially similar to a single dose of 80 per cent of the LD50 (0.20 mM/Kgm) given at one time. Kymographic records of such experiments are presented in figure 1.

The character of both the immediate and the slow blood pressure changes was not influenced by bilateral vagotomy performed 24 hours prior to the injection of

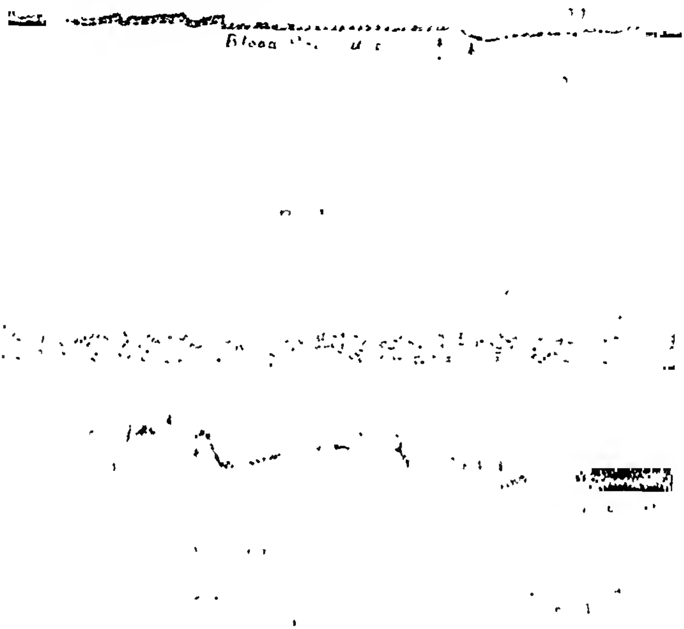

FIG. 1. THE EFFECT OF SMALL (UPPER RECORD) AND LARGE (LOWER RECORD) DOSES OF BAL ON THE ARTERIAL BLOOD PRESSURE OF THE CAT

BAL, nor stimulation of the vagus during injection, while vagal slowing is normal during BAL intoxication. Although respiratory depression accompanies poisoning, impaired ventilation likewise appears to exert little influence in BAL poisoning for artificial respiration did not modify the response of the blood pressure to BAL.

ELECTROCARDIOGRAM. The electrocardiogram (Lead II) was obtained in three anesthetized cats. Doses of BAL of approximately 40 per cent of the LD50 were injected intravenously at intervals of 10 to 15 minutes in total doses sufficient to produce marked respiratory distress. Several changes in the electrocardiogram resulted, varying in degree with the dose of BAL. Heart rate

changes were slight and varying. Decrease in the R-wave voltage, deepening of the S-wave, elevation of the S-T segment, occasional ectopic beats, brief periods of ventricular tachycardia and lowering of the T-wave were observed at different times in the three cats. Figure 2 shows representative sections of typical tracings. Until excessively high doses (over 100 per cent of the LD50) were administered the changes in the electrocardiogram were minimal in character and could well be ascribed to increased irritability and diminution of the voltage complex

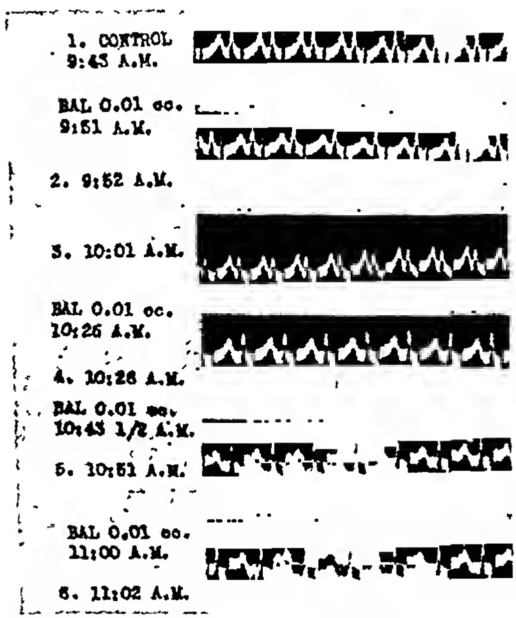

FIG. 2. SAMPLE ELECTROCARDIOGRAPH TRACINGS SHOWING THE CHANGES PRODUCED BY BAL IN ONE ANIMAL
(Cat 115; Lead II)

secondary to myocardial anoxia. Some direct action of BAL on the myocardium doubtless exists, but can not be responsible for the fall in blood pressure.

PERIPHERAL RESISTANCE. The cause of the continued fall in blood pressure following large intravenous doses was the first object of inquiry. Because of the rapid fall of pressure it was throught that vaso-dilatation might be a factor. In order to study the peripheral resistance the principle employed by Bartlett (3) was utilized. A burette was connected by means of a centrally directed cannula to a side-branch of the femoral artery, usually the perineal artery. During the measurement of peripheral resistance a bull dog clamp was placed on the femoral artery just cardiad to the side branch. The peripheral resistance was measured as the time in seconds required for 3 cc. of saline to enter the leg from the burette

under a constant pressure equal to the carotid artery pressure. A typical experiment performed on an anesthetized cat is presented in figure 3. As can be seen in this figure, the increase in peripheral resistance following the injection of BAL became so great that the inflow of saline was completely blocked. At the same time a marked and persistent fall in mean arterial pressure was recorded. Following the death of the animals a rapid fall in resistance toward normal levels occurred. This indicates that the mechanism of increased peripheral resistance is functional in character and not due to some adventitious mechanical blockage.

A further series of experiments were carried out in an attempt to determine the primary site of action of BAL. Three cats were prepared by transecting the spinal cord at the C8 level, respiration being maintained artificially. The same

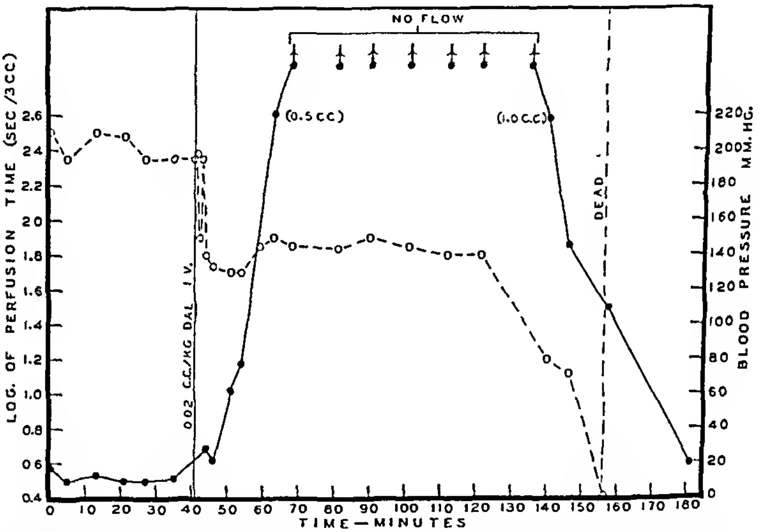

FIG. 3. THE EFFECT OF INTRAVENOUS BAL ON THE RESISTANCE TO FLOW THROUGH THE FEMORAL ARTERY

Open circles, arterial pressure; solid circles, perfusion rate

increase of peripheral resistance followed the intravenous injection of BAL in these animals as in normal animals. This indicated that the site of the mechanism involved in the production of the increased peripheral resistance does not involve the medullary vasomotor centers.

Further light was thrown on the subject by two similar experiments one of which is presented graphically in figure 4. In these experiments both femoral arteries of a cat were cannulated and peripheral resistance was determined independently in each leg by means of the Bartlett technique. One leg was perfused with physiological saline and the other was perfused with BAL, 0.08 and 0.05 per cent solution in the two experiments respectively in saline. In the case of the leg perfused with normal saline no increase in peripheral resistance was

detected but in the leg perfused with BAL-saline an increase rapidly developed to the point of complete obstruction. The figures in parentheses in figure 4 are the actual volumes of perfusates which were introduced. A striking reduction in flow resulted at first, followed by a return to normal. Continuation of the perfusion resulted in a less marked increase in peripheral resistance, which again reverted to normal and was again followed by a relatively slight rise and fall in peripheral resistance. Presumably the reason for the return to normal during perfusion is that very small amounts of BAL-saline could be introduced during the period of maximum obstruction. That the action of BAL on the peripheral resistance is transitory is well demonstrated. During this period the systemic blood pressure was well maintained, a point which will be further considered be-

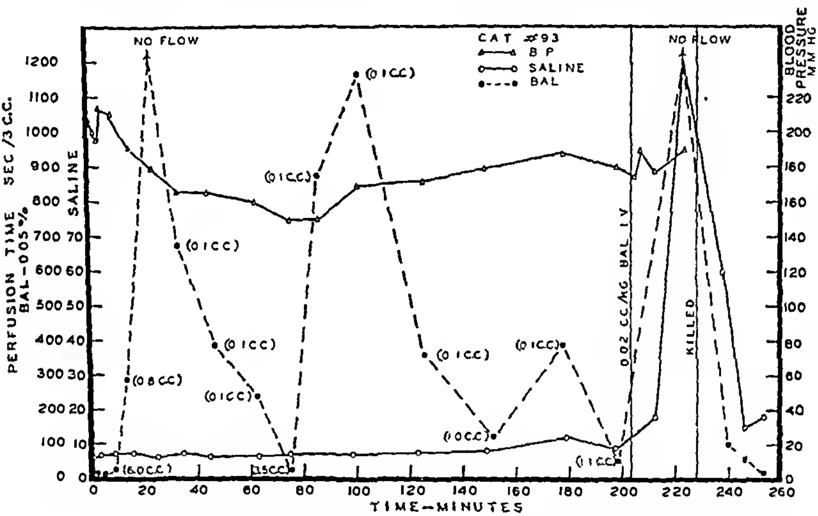

FIG 4. EFFECTS OF BAL ON FLUID PERFUSED THROUGH FEMORAL ARTERY (SOLID CIRCLES) Opposite leg (open circles), upper curve (triangles) arterial pressure

low. Following the third rise in peripheral resistance a dose of approximately 80 per cent of the LD50 (0.20 mM/Kgm.) was injected in the jugular vein. A prompt increase in peripheral resistance to the point of complete obstruction in *both* legs followed the injection. After the animal was killed with ether, the resistance returned to nearly normal levels. Taken in conjunction with data on the isolated leg and spinal cat experiments these observations indicate that this action of BAL is exerted peripherally and not through central mediation.[4]

LIMB VOLUME. The only reasonable explanation for such a pronounced reversible phenomena must be on the basis of a physiological mechanism and the

[4] Dr. Philip Hitchcock while a member of this Department carried on a series of experiments on the isolated cat leg which confirmed the results obtained in the intact animal. A separate report will be made upon this aspect of the study.

only important physiological cause of increased peripheral resistance is arteriolar constriction. If such were the case in BAL intoxication, a marked reduction in limb volume should be observed. Figure 5 presents the changes in volume of an intact cat's leg enclosed in a water plethysmograph kept at a constant temperature. Indices of peripheral resistance determined on the opposite leg by the usual method are also plotted on this figure. Following the injection of BAL a distinct decrease in leg volume paralleled the increase in peripheral resistance. As soon as peripheral resistance reached a high point the leg volume decreased sharply, providing further evidence of a marked arteriolar constriction.

Arteriolar diameter. In order further to confirm this concept direct inspection of the arterioles was carred out in 4 cats under anesthesia. In two of

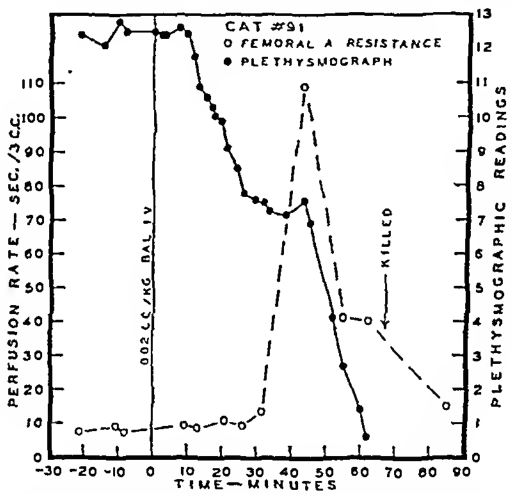

Fig. 5. The Effect of BAL (0.02 cc.) on Leg Volume

The fall in the readings (solid circles) represents a decrease in leg volume. Open circles, perfusion rate (Bartlett technic) in opposite leg.

these the carotid artery pressure was recorded. The sartorius muscle was freed along its length, mineral oil was liberally applied to prevent drying and a bent "lucite" (methyl methacrylate) rod with optically semi-finished ends was passed under the muscle with the flat end of the rod pressed gently against the muscle. Care was exercised to prevent undue stretching of the muscle. A moderately intense beam of light was focused on the free end of the rod and, due to the transmitting properties of "lucite," the light followed the bend and passed perpendicularly through the muscle. Thus a strong, cool trans-illumination was obtained.

Through a dissecting microscope a clear view of arterioles and the accompanying venules could be had in this manner. Control injections of epinephrine made into the jugular vein produced almost instantaneous constriction of the arteriole under inspection which rapidly diminished in diameter until it almost disappeared

from view. Similar injections of BAL in doses varying from 40 to 80 per cent of the LD50 produced an equivalent degree of arteriolar constriction, but differed from epinephrine in that a delay of 6 to 8 minutes preceded the onset of the constriction. The constriction was preceded by the usual fall in systemic arterial pressure.

SPLANCHNIC CIRCULATION. I. *Arterial.* A fall of systemic arterial pressure in the presence of marked peripheral arteriolar constriction might occur were the splanchnic vascular bed to react to BAL to a much lesser degree than the peripheral vascular bed, thus permitting pooling of blood. The Bartlett technique was applied to the superior mesenteric artery by cannulating a small side branch. The intravenous injection of 50 to 60 per cent of the LD50 dose of BAL produced no increase whatever in the resistance of the splanchnic bed, in fact, as illustrated in figure 6, there was a distinct decrease in splanchnic resistance in both of the two

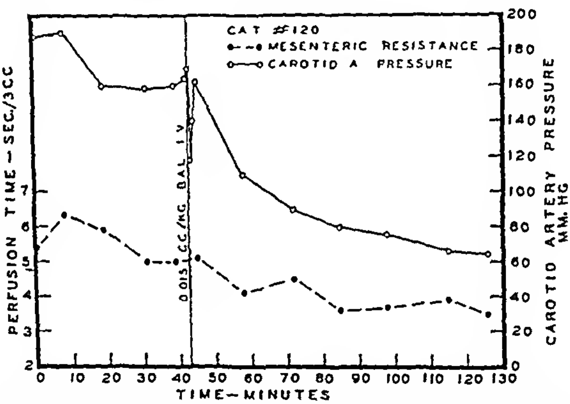

FIG. 6. THE EFFECT OF BAL (0.015 cc.) ON THE PERFUSION RATE THROUGH A BRANCH OF THE MESENTERIC ARTERY (LOWER CURVE). UPPER CURVE SHOWS CHANGES IN ARTERIAL PRESSURE

cats studied. A parallel fall in blood pressure occurred. However, no marked degree of visceral congestion was observed, and this did not seem adequate to account for the fall in pressure.

SPLANCHNIC CIRCULATION. II. *Venous.* To establish the actual degree of pooling the portal vein pressure and the vena caval pressure were recorded simultaneously with the arterial pressure. Cannulae connected to a water manometer were inserted into the splenic vein directed toward the liver in 6 cats. Following the intravenous injection of 20 to 80 per cent of the LD50 a distinct rise in portal venous pressure was observed which lagged somewhat behind the falling carotid artery pressure. Furthermore, the portal pressure reached a definite peak and then declined, although systemic arterial pressure continued at a low level. In two of these animals the vena caval pressure was measured similarly through a cannula introduced into a renal vein, the tip projecting into the vena cava. During the period of rising portal pressure there was a decline in vena cava pres-

sure as well as the customary fall in systemic arterial pressure. Figure 7 presents a plot of the three pressures taken simultaneously in a cat.

Were pooling of blood in the splanchnic areas of importance in the production of the fall of arterial pressure, the rise in portal vein pressure would be expected to persist throughout the period of the depression of arterial pressure. That this is not the case is readily apparent. Therefore, although considerable pooling of blood in the viscera does occur, which may contribute in the fall in arterial pressure, it is unlikely that it is a primary factor, especially since the onset of increased portal venous pressure lags several minutes behind the falling arterial pressure.

Intrahepatic portal vein resistance. An increase in hepatic resistance was considered as a possible factor in the production of increased portal vein pres-

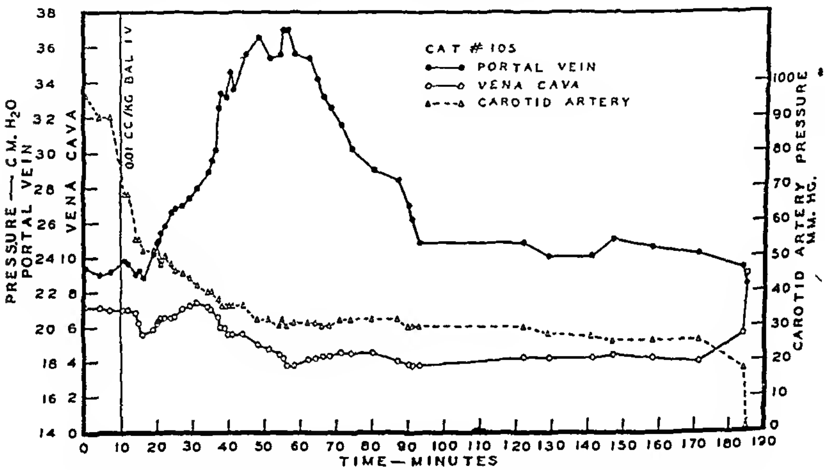

Fig. 7. Pressure Changes in the Carotid Artery, the Portal Vein and the Vena Cava, Resulting from the Intravenous Injection of 0.01 cc. BAL

sure. The Bartlett technique was applied to the hepatic portal circulation of the cat but no change in hepatic resistance followed the injection of BAL. Similar results were obtained by Dr. Hitchcock with isolated, perfused rabbit liver. Therefore, venous constriction in the liver is not the cause of increased portal vein pressure.

Blood volume. At this point the attack was shifted to a different aspect of the problem; the behavior of the capillary bed. Earlier in the work a marked increase was observed in the cell volume of the blood as indicated by elevated hematocrit readings. At that time it was set aside as a logical consequence in such severely depressed animals poisoned with BAL. The phenomenon was re-examined in both intact and anesthetized animals. A study of hematocrit and hemoglobin changes in two unanesthetized cats is presented in figure 8. A rather high degree of hemoconcentration is revealed by the increase in cell volume and

hemoglobin levels following the injection of BAL. The control animal showed no significant change, despite the withdrawal of blood, demonstrating that the effect is due to BAL.

Changes in plasma and total blood volume are of considerable significance and therefore a more precise procedure was utilized. In a series of 4 experiments performed on anesthetized cats ("Dial") 5 mgm./kgm. of the blue dye T-1824 was injected intravenously and the concentration of the dye at regular intervals thereafter was determined with a photo-electric colorimeter. Simultaneous determinations were made of the hematocrit level. The results of these determinations are of special interest and are tabulated below. (See table 1.) A graphic plot of a typical experiment is presented in figure 9. The blood volume calculated on the basis of the hematocrit readings is found to *decrease* by

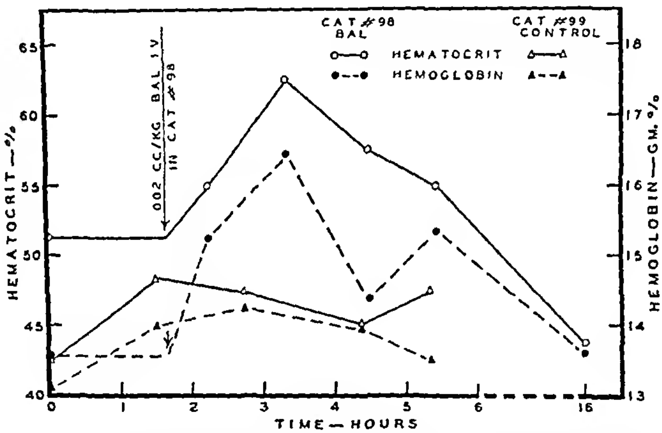

FIG. 8 CHANGES IN BLOOD CONCENTRATION IN A CAT INJECTED WITH 0 02 cc BAL

The two upper curves represent the hematocrit and hemoglobin reading in the treated cat, and the two lower curves in the control animal.

30 per cent, whereas if the calculation is based upon the changes in dye concentration, the blood volume is found to *increase* by 27 per cent. This paradox is presumably to be explained by the fact that the protein-dye complex, which is the form in which the dye exists in the plasma according to Rawson (4), escapes in large measure from the blood stream after poisoning by BAL. Since the dye is ordinarily non-diffusible, the entire protein-dye complex must pass out of the circulation, presumably through the capillaries.

That such is the case is clearly evidenced by direct observation of an animal which has received 5 mgm./kgm. of the dye prior to an injection of BAL. During the period before the injection of BAL the animal acquires a slate-gray coloration about the oral mucosa, nipples and sclerae. Within 15 to 30 minutes after the injection of BAL a striking change occurs. These areas become intensely and brilliantly blue, even the retina being deeply stained.

The increase in capillary permeability which follows the administration of BAL is evidently extreme for a state of shock produced by intestinal trauma results in only slightly increased staining after T-1824. It may be assumed that

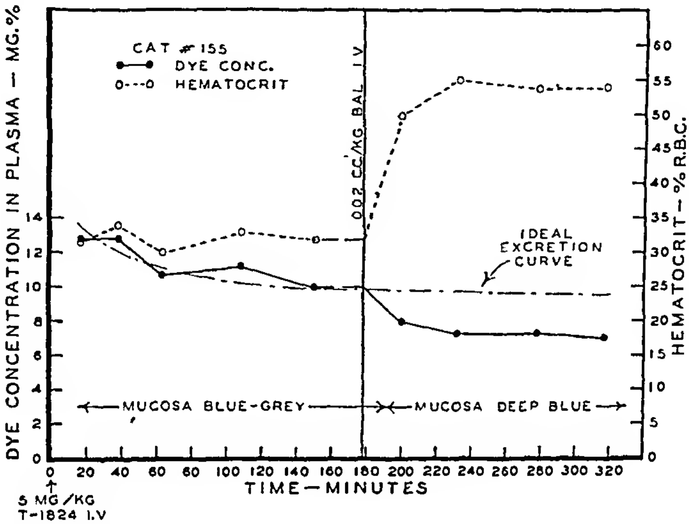

FIG. 9. EFFECT OF 0.20 mM (0.02 cc.) BAL ON THE PLASMA CONCENTRATION OF THE "NON-DIFFUSIBLE" DYE, T-1824 AND ON THE PER CENT RBC IN THE BLOOD

TABLE 1.

Changes in cell volume and dye concentration in the blood of cats receiving a single intravenous injection of 0.20 mM BAL

CAT NO.	CELL VOLUME, %		CONCENTRATION OF DYE MG. PER 100 CC.	
	Control	After BAL	Control	After BAL
144	36	45		
151	16	29	9.6	8.0
154	42	51	10.5	6.0
155	32	54	10.5	7.3
Average..............	31.5	44.8	10.2	7.1
Percentage change.....		+27		−30

the changes observed are the result of the direct action of BAL on the capillaries, apart from effects which may be secondary to other circulatory actions.

LYMPH PRODUCTION. In view of the evidence just presented that BAL causes a marked loss of plasma protein through the capillary bed a series of experiments was performed in the cat to determine the effect of BAL injections on the pro-

duction of lymph. The earlier determinations were made upon the flow from the thoracic duct and later, upon the flow from the superior cervical channels. The technique was a modification of that employed by McCarrell (5). Following exposure of the lymphatic channel under investigation an amount of chlorazol pink sufficient to prevent clotting of blood and lymph was injected intravenously. In the case of the superior cervical lymphatic channel the jaw of the animal was rhythmically opened and closed by an electric motor at the rate of 12 times per minute. In this manner a constant lymphatic flow was obtained. A cannula was inserted into the duct and tied in place. A system containing normal saline arranged to displace a more concentrated salt solution from a reservoir was connected to the cannula. A drop of this latter solution made an electrical contact

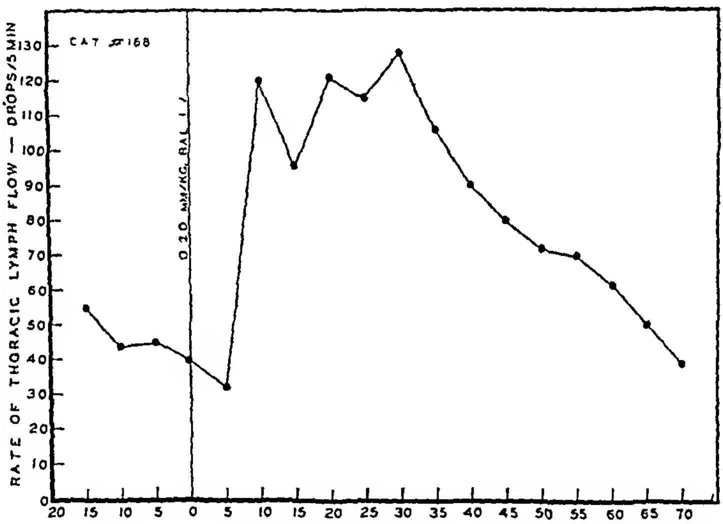

FIG. 10. LYMPH FLOW FROM THORACIC LYMPH DUCT FOLLOWING THE INTRAVENOUS INJECTION OF BAL

and actuated a signal magnet recording on a kymograph. Five determinations were made on the thoracic duct flow and in all a marked increase in the lymph flow was obtained following the intravenous injection of BAL. A representative experiment is charted in figure 10. Even after a systemic blood pressure had fallen to shock levels with an attendant decrease in filtration pressure the production of lymph was greater than during the control period. However, in view of the increased portal pressure also produced by BAL it was at first thought that the increase in lymph flow from the thoracic duct might have been due to the marked increase in filtration pressure. However, that this was not the cause was established by the finding that the lymph flow from the superior cervical channels was equally affected. Figure 11 presents the results of such an experiment.

EFFECT OF MINUTE DOSES OF BAL. I. *Intravenous.* As noted above the repeated administration of small doses of BAL did not cause a fall in arterial

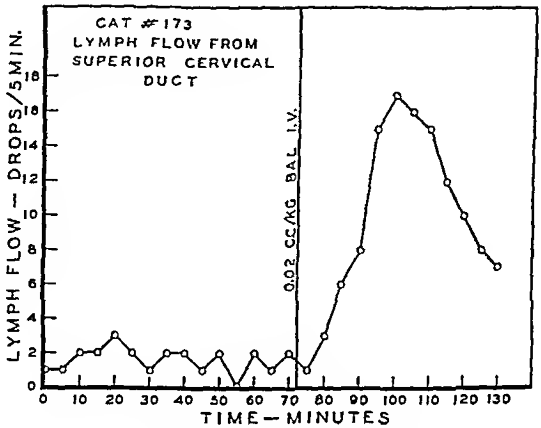

FIG. 11. LYMPH FLOW FROM SUPERIOR CERVICAL DUCT FOLLOWING THE INTRAVENOUS INJECTION OF BAL

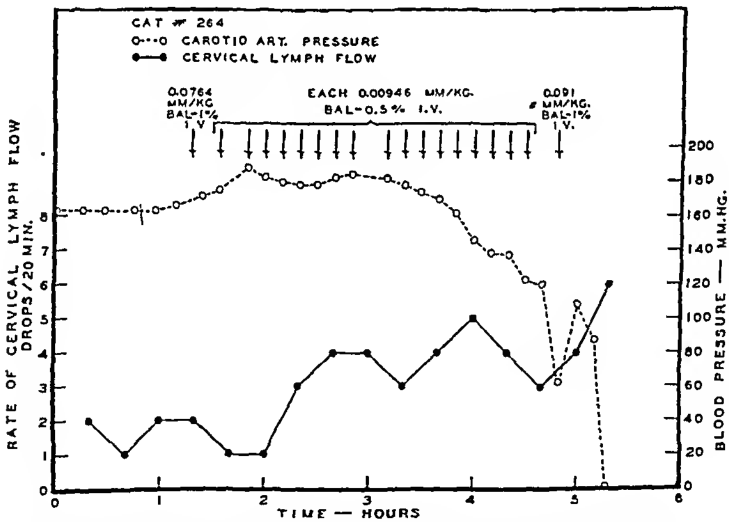

FIG. 12. CHANGES IN THE ARTERIAL PRESSURE AND CERVICAL LYMPH FLOW IN A CAT RECEIVING INTRAVENOUSLY REPEATED SMALL INJECTIONS OF BAL

Towards the end of the experiment a large intravenous dose was given

pressure although they produced other changes characteristic of BAL. The production of extreme vasoconstriction should ordinarily result in an elevation of

blood pressure. That this did not occur was probably the result of the markedly increased capillary permeability and the attendant reduction of blood volume. However, that it might be possible to dissociate to some degree these two actions of BAL is indicated by the earlier observation. To establish this point more adequately blood pressure changes were followed in 5 cats in which the usual dose (0.20 mM/kgm.) was given in 20 divided doses at intervals of from 5 to 10 minutes. The blood pressure was well sustained in all but one animal and in all exceeded the preinjection level at some time during the experiment. Figure 12 presents such an experiment, together with a record of the lymph flow from the superior cervical channel. As can be seen from this figure, the lymph production increased well in advance of the decline in systemic arterial pressure. It is apparent that small doses of BAL may produce an arteriolar constriction which elevates the systemic blood pressure to a significant degree prior to the development of increased capillary permeability. As capillary permeability is increased with the continued administration of BAL there is a decline in the systemic arterial pressure despite the continuation of arteriolar constriction.

EFFECT OF SMALL DOSES OF BAL. II. *Percutaneous application.* BAL was first used as a chemical and pharmacological skin decontaminating agent against arsenical vesicants in chemical warfare. The effect of BAL on the skin, *per se*, and its effectiveness for this and other purposesneed not enter into this discussion. It was of importance, however, to determine to what degree the characteristic circulatory changes described above would develop following percutaneous administration of BAL.

Anesthetized cats were clipped over the entire ventral surface, including the tail, and BAL was applied evenly over this surface in doses varying from 1.50 to 3.00 mM/kgm. In 4 cats, one of which showed a slight temporary drop related to the anesthesia, the arterial pressure was well maintained. In one of 3 animals there was a slight increase in peripheral resistance as measured by the Bartlett technique. In two animals in which the lymph production was recorded from the cervical channels and two in which thoracic duct flow was recorded there was a questionable increase in flow amounting to not more than 50 per cent of the control values. In several animals injected with T-1824 prior to the application of BAL evidence of severe capillary damage confined to the areas of BAL application was readily visible. In all the effects studied, however, it was evident that even large doses of BAL applied to the skin have a very minor action in comparison with that observed following intravenous injection.

DISCUSSION. The experimental data presented above support the view that the major actions of BAL on the cardio-vascular system are twofold (6), a marked increase in permeability resulting from the action of BAL on the capillary wall and (2), a peripheral arteriolar constriction as the result of the action of BAL directly on the arteriolar structures. A third factor, of a negative character, is the absence of vasoconstriction in the splanchnic arterioles. The various changes in the circulatory system which have been described are probably secondary to these three phenomena.

The clinical importance of an understanding of the toxic as well as the therapeutic actions of such a useful drug as BAL is evident. Toxic manifestations following intramuscular injections of BAL in man have been described (6). In general, the cardiovascular changes are limited to the production of a transient hypertension, which presumably corresponds to the results described following small repeated intravenous doses or percutaneous administration in cats.

From a pharmacodynamic point of view BAL presents a very interesting phenomenon. The action of potent vasoconstricting agents is customarily regarded as general throughout the vascular bed. BAL produces a decidedly atypical reaction in that peripheral arterioles are markedly constricted while splanchnic arterioles are unaffected. The action of BAL on the peripheral arterioles has been established to be on the arteriolar structure and it may be assumed that the sensitive component of the peripheral vessels is absent from the splanchnic vessel.

SUMMARY

1. BAL administered to cats by vein results in a fall of the systemic and pulmonary arterial pressure, a fall of the venous pressure, and a rise of the portal pressure.

2. In very small repeated intravenous doses or by percutaneous application, BAL causes a rise in blood pressure.

3. There is a marked rise in the peripheral resistance of the limb vessels during the period of falling blood pressure but none in the vessels of the liver or splanchnic area.

4. Following BAL the hematocrit reading is increased while at the same time the concentration of injected dye in the blood is reduced. This paradoxical result is probably due to the loss of the dye from the blood stream following BAL.

5. BAL resulted in a marked increase in the rate of lymph flow from the thoracic and cervical ducts.

6. It is concluded from these experiments that BAL has a primary action resulting in a reversible constriction of certain peripheral arterioles. This action is reflected in a rise in blood pressure from small doses. Larger doses result in capillary damage and the development of the signs of peripheral vascular failure. The other changes observed are probably secondary to these two main actions.

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EFFECT OF DITHIOLS AND OTHER ENZYME INHIBITORS ON BLOOD VESSELS[1]

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In a study of the pharmacology of BAL (2,3-dimercaptopropanol in the intact cat, it was found to produce a complex group of changes in the circulation involving a rise of the blood pressure with minute doses, but with larger doses a fall to shock levels. It was found that these represent a direct arterial constrictor action as well as a direct toxic injury of the capillaries (1). It was also observed in the intact animal that the constrictor response of the femoral artery was not shared by the mesenteric artery.

A series of experiments has been carried out to explore further the action of the dithiols on blood vessels, and, in view of the fact that BAL interferes with enzyme functions (2), to compare their actions with those of other enzyme inhibitors in isolated perfused organs. The results form the subject of the present report.

METHOD. The organ was perfused by a modification of the method of Katz, Paine, and Tiller (3). This method involves an almost constant flow, variation in resistance being shown by a change in perfusion pressure. The inlet arm was provided with a stop-cock to which two Marriott bottles were connected, one containing the drug and the other, the control perfusion fluid. The rate of flow was adjusted to a pressure of from 10 to 15 cm. by means of the stop-cock. The perfusion fluid was usually a buffered Ringer's solution with a pH of 7 2. In a few cases it contained acacia or cat's blood.

The organs perfused were the hind limb (cat), liver (cat, rabbit), kidney (cat), and heart (cat).

The agents used were the dithiols, 2,3-dimercaptopropanol (133), hexanedithiol-1,6 (139), 2,3-dimercaptopropyl ethyl ether (293); sodium cyanide; sodium azide; sodium malonate; sodium iodoacetate; sodium fluoride; and sodium arsenite.

When a change in resistance to flow failed to occur, the responsiveness of the vessels was tested by epinephrine. That an increase in resistance to flow was due to constriction rather than to edema was indicated by the fact that the plethysmograph showed a reduction in volume and further by the fact that the resistance fell off as the preparation deteriorated. The same was observed in the intact animal after death.

RESULTS. Several typical experiments have been charted for illustration.

Figure 1 shows the effect of BAL perfused through the isolated rabbit's liver. There is no significant change in resistance.

Figure 2 shows the marked increase in resistance when BAL is perfused through the isolated cat's limb. The effect can be reversed by washing out and then repeated. Repetitions do not yield equal constriction, the exact reason for which has not been established.

[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

Figure 3 shows a comparison of three dithiols. 133, 139, 293, on the resistance to flow in the isolated cat's limb. It may be noted that 133 (BAL) produced a

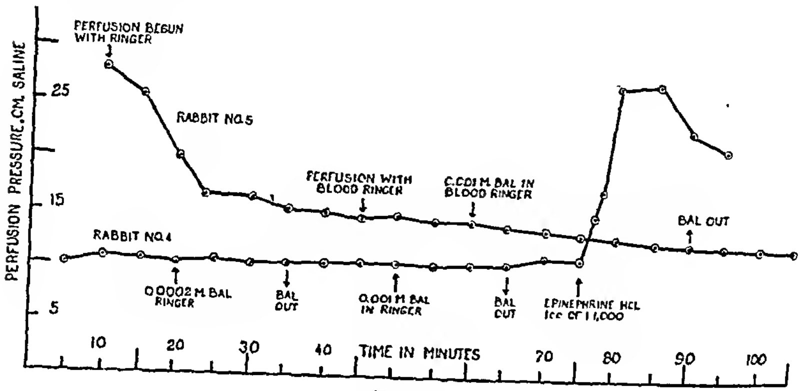

FIG. 1

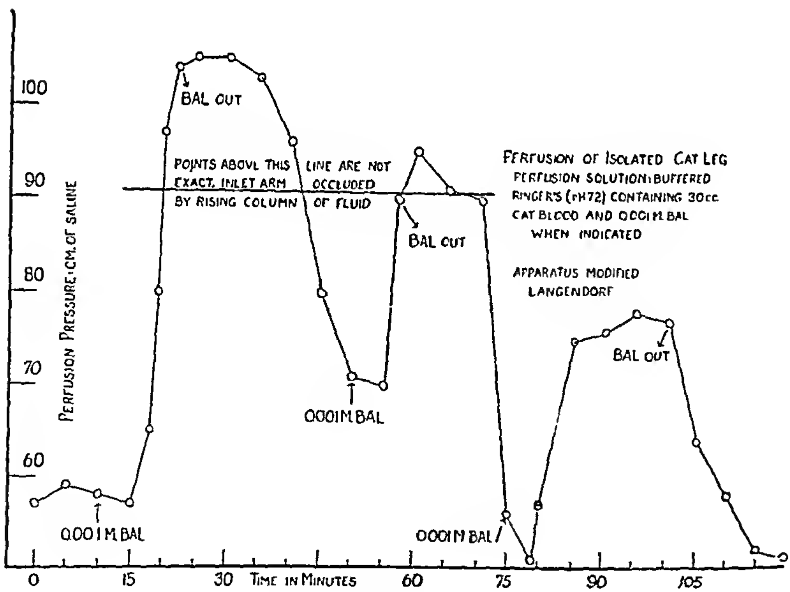

FIG. 2

marked reversible constrictor action, while 139 and 293 produced a marked but irreversible constrictor action.

Figure 4 compares the effects of 293, sodium cyanide, and sodium fluoride on

resistance in the isolated cat's limb. All three produced marked constriction, but only that of sodium fluoride was reversible.

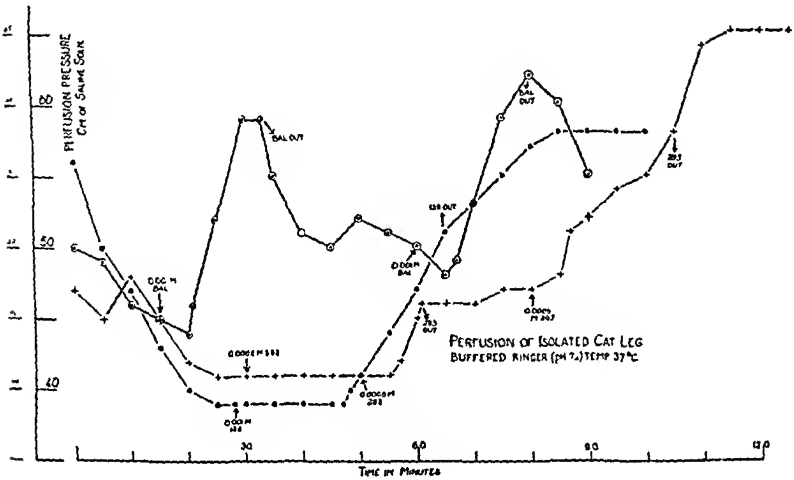

FIG. 3

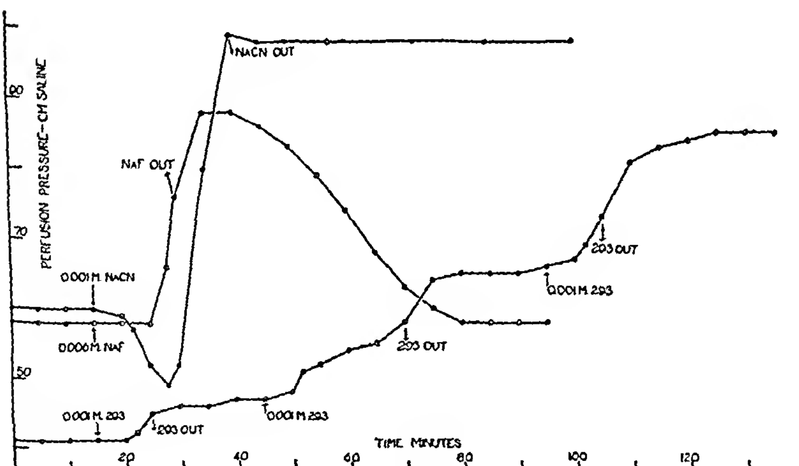

FIG. 4

The results of all 53 experiments have been summarized in table 1. Blood vessels of different organs behave differently to enzyme inhibitors. BAL which is an intense constrictor of the femoral arterioles is without effect on the renal, hepatic,

or coronary arterioles. Closely allied compounds of the dithiol group exert different actions on the femoral arterioles; one produced a reversible constriction; and two others an irreversible constriction. While one dithiol was without effect on the hepatic vessels, another caused constriction. Several other enzyme inhibitors were found to produce irreversible constriction of the femoral arterioles, cyanide, azide, malonate, iodoacetate; some, reversible constriction (fluoride); still others, no effect (arsenite). Some factor other than simple anoxia appears to be responsible for the constrictor action as evidenced by the absence of constriction in the experiment performed with nitrogen replacing oxygen in the

TABLE 1

Effect of enzyme inhibitors on vascular resistance in isolated perfused organs

DRUG	ORGAN	NO. OF EXPERIMENTS	CONCENTRATION	INCREASED RESISTANCE	REVERSIBLE
Dithiol-133	Femoral—cat	12	0.0005 M–0.001 M	+	+
Dithiol-139	Femoral—cat	1	0.001 M	+	0
Dithiol-293	Femoral—cat	3	0.001 M	+	0
Sodium cyanide	Femoral—cat	6	0.001 M	+	0
Sodium azide	Femoral—cat	1	0.004 M	+	0
Sodium malonate	Femoral—cat	1	0.001 M	+	0
Sodium iodoacetate	Femoral—cat	2	0.001 M	+	0
Sodium fluoride	Femoral—cat	3	0.004 M	+	+
Sodium arsenite	Femoral—cat	2	0.001 M	0	–
Dithiol-133	Portal—cat	6	0.0002 M–0.001 M	0	–
Dithiol-133	Portal—rabbit	5	0.0002 M–0.001 M	0	–
Dithiol-139	Portal—rabbit	1	0.005 M	+	+
Sodium cyanide	Portal—rabbit	1	0.005 M	+	+
Sodium fluoride	Portal—rabbit	1	0.005 M	+	+
Sodium iodoacetate	Portal—rabbit	2	0.005 M	0	–
Dithiol-133	Kidney—cat	3	0.001 M	0	–
Dithiol-133	Coronary—cat	2	0.001 M, 0.005 M	0	–
Nitrogen	Femoral—cat	1		0	–

perfusion. The fact that the flow ceased completely during some of the perfusions with BAL indicates that constriction occurred in the vessels of the skin as well as the muscles.

COMMENTS. Chemical agents acting on the smooth muscle of the blood vessels fall, broadly speaking, into two classes, those in which the action is determined by nature of the nerve supply, and those in which the action is independent of the nature of the nerve supply. Epinephrine is an example of the first class. It constricts the skin vessels but dilates the coronary vessels. It simulates, as is well known, electrical stimulation of the sympathetic nerves. Pituitrin and the

nitrites are examples of the second class. Pituitrin constricts and the nitrites relax all blood vessels without regard to the innervation. There is evidence in the literature in relation to pituitary solution, that the intensity of its action is not the same on all blood véssels, but the evidence seems fairly satisfactory that all blood vessels are constricted by this material (4). Qualitative differences in the response of vessels from different organs are commonly assigned to differences in innervation.

In the present experiments, evidence has been obtained which indicates that in the blood vessels of different organs, different chemical mechanisms operate, giving rise to qualitative differences in response to agents, quite apart from those related to the innervation.

SUMMARY AND CONCLUSIONS

1. In a series of perfusion experiments with isolated organs, several enzyme inhibitors were found to produce vascular constriction.

2. The results show that vessels of different organs behave differently to various enzyme inhibitors, and to closely allied members of the dithiol group.

3. Evidence is presented to the effect that the vascular response of different organs to enzyme inhibitors shows qualitative differences without relation to the innervation.

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THE ACTION OF 2,3-DIMERCAPTOPROPANOL (BAL) AND RELATED DITHIOLS ON THE ISOLATED SKELETAL MUSCLE OF THE FROG[1]

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It has been demonstrated by several investigators that BAL inhibits the oxygen uptake when added to tissue slices. This was shown for liver slices and other tissues by Rhoads and Kensler (1) and for bone marrow by Warren (2). In the present study corroborative evidence was sought through an investigation of the influence of BAL on the functional changes occurring in the isolated sartorius muscle of the frog. This preparation has the advantage over tissue slices in the fact that changes in oxygen utilization or lactic acid production may be correlated with the contractile response, and this provides a control against the possibility that a falling off in oxygen consumption might be related to non-specific cellular damage.

METHODS. For the measurement of oxygen uptake the myothermic technic of A. V. Hill, as employed in other studies from this laboratory (3), was utilized. The experiments were carried out on the isolated frog (*R. pipiens*) double sartorius muscle preparations at 21.5°C. After dissection, the muscles were mounted on a thermopile and soaked in phosphate buffered (pH 7.3) oxygenated Ringer's solution for about one hour, during which time the temperature of the thermopile and water bath, which was stirred by bubbling air, became stable. Then the Ringer's solution was removed, 5 to 10 minutes were allowed to elapse for thermal equilibrium to be attained, and the heat measurements were carried out in an atmosphere of oxygen. Between single or groups of measurements, the muscle was reimmersed in the Ringer's or BAL-Ringer's solution. In most cases, the heat from 3 twitches initiated by maximal break induction shocks delivered 1 second apart was recorded as a single determination. However, in the case of large muscles, heat production from a single twitch was sufficiently great to allow accurate measurement. Simultaneously, the twitch tension was recorded photographically. A high frequency oscillator was used in calibrating the muscle preparation for the determination of the initial heat.

After a series of control twitches, the muscle was immersed in BAL of suitable concentration, freshly dissolved, in Ringer's solution for periods of 1 to 2 hours; exposures to BAL were repeated, in most cases, once or twice.

The second group of experiments was carried out to determine the influence of BAL on the production of lactic acid. Two pairs of double frog sartorius muscles were isolated and immersed in 25 cc. oxygenated, phosphate buffered (pH 7.3) Ringer's in separate chambers. They were stimulated by condenser discharges every half minute and the contractions were recorded on a kymograph. After a control period of 1 hour, BAL was added to one chamber to give the desired concentration and the experiment continued for from 4 to 4½ hours.

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[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] Present address: Department of Pharmacology, Yale University Medical School, New Haven.

[3] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

Before stimulation was begun, 1 to 2 cc. samples of the Ringer's were withdrawn and diluted to 10 cc with 5 per cent trichloracetic acid for the lactate determinations. This procedure was repeated at ½ to 1 hour intervals thereafter throughout the experiment. In the majority of the experiments, the volume of Ringer's solution removed was immediately replaced by an equal volume of fresh Ringer's solution. At the end of the experiment, the muscles were pressed gently between filter papers and their "wet weight" obtained. The lactate determinations were carried out at the Memorial Hospital by C. J. Kensler. The p-hydroxydiphenyl method as modified by Barker and Summerson was employed

TABLE 1

The effect of BAL on the heat production of contracting frog muscle

EXPT., 1942	BAL CONC	CONTROL*	AFTER BAL					
			1st exposure			2nd exposure		
		Delayed heat (initial heat = 100)	Time of exposure	Initial heat in % of control	Delayed heat (initial heat = 100)	Time of exposure	Initial heat in % of control	Delayed heat (initial heat = 100)
			min.			min		
11/10	1:5000	92	45	122	34	80	108	0
11/12	1:5000	27	70	89	5			
11/11	1:5000	41	115	113	18	130	105	2
11/18	M/1000	22	90	110	22			
11/19	M/1000	79	80	115	60			
11/20	M/1000	26(?)	95	100(?)	9	220	74	0
11/23	M/1000	61+	95	116	31	155	108	5
11/24	M/500	63	60	113	22	120	30	15
11/25	M/500	39	60	124(?)	25	120	36	2
11/28	M/500	70	70	106	24	135	61	20
12/3	M/1000	102	60	103	26	105	98	33
12/4	M/1000	83	70	145	16	120	99	22
12/8	M/500	86	65	146	10		96†	19†
12/9	M/2000	79	200	125	47			
Average		62.1		116	25.3			13.5

* All determinations based on heat from 1 to 3 twitches; if more than one twitch used, they were spaced 1 second apart.

† After 1 hour washing and soaking with fresh Ringer's following 65 minutes in BAL M/500.

The muscles in experiments 12/3 and 12/4 were subjected to a third exposure to BAL, after which in both experiments the delayed heat fell to 8% of the initial heat.

RESULTS. The results dealing with the influence of BAL on the heat production of the frog's sartorius muscle are summarized in table 1. In M/2000 to M/500 concentration, BAL appreciably lowered or completely abolished the oxidative heat. In general this effect increased as the exposure time was increased. In 2 experiments, after the initial decrease, there was a small subsequent change in the opposite direction, but this may not be significant. It should be noted that the measurements of delayed heat include a small amount of energy from anaerobic processes amounting to from 10 to 20 per cent in a series

of twitches (4). However, the evidence indicates that the percentage would be lower when fewer twitches are involved as in the present experiment. Thus a small quantity must be subtracted from the measurements of delayed heat to obtain the true value of the heat due to oxidations. Thus the figures for the delayed heat in the table probably signify complete inhibition of oxidation when they fall below about 10 per cent of the initial heat.

During the first hour or two of exposure to the BAL solution there was in all but one instance an increase in the energy (initial heat) liberated during contraction which in the 14 experiments listed in the table averaged 16 per cent. In those experiments in which the twitch tension was recorded there was a corresponding increase in the mechanical response, indicating that BAL did not influence the "efficiency" of contraction. These changes are similar to those occurring when the muscle is placed in an environment of nitrogen and presumably have the same significance, viz., are characteristic of contraction under anaerobic conditions. After long exposure to BAL the twitch tension declines and the muscle finally becomes inexcitable.

In M/500 concentration, BAL caused lactate to accumulate in the Ringer's solution surrounding the muscle in the presence of oxygen. The results of the 3 experiments are presented graphically in figure 1. The results are expressed as gamma of lactic acid per gram of muscle in the surrounding Ringer's solution, disregarding the water content of the muscle mass, which was small (166 mg. to 330 mg.) compared with the volume of Ringer's used (25 cc.). During the control period, one out of six preparations showed a small gain in lactate; also, one showed a small lactate concentration prior to beginning of stimulation. It is clear that the lactate concentrations are increased and tend to reach a maximum within 1 or 2 hours. In those experiments in which the muscles were continuously stimulated at the rate of twice a minute there was a gradual fall in twitch tension, sometimes preceded by a short period of augmentation. In two experiments, lactate accumulation was quite pronounced at a time when the functional state of the muscle was good as reflected in a nearly normal twitch tension. In the single experiment performed at 16.5°C., the rate of lactate accumulation reached its maximum more slowly than did that of the other experiments at 20.5°C.

A few observations were made on the influence of two related dithiols, the ethyl ether of BAL and hexanedithiol-1,6, on the oxidative recovery heat of frog muscle. The effects, summarized in table 2, are similar to those of BAL.

Exposure for one hour to 0.001 and 0.005 M solutions resulted in inhibition of the oxidative recovery heat which was practically complete and there was no indication of reversal when the muscle was subsequently soaked in fresh Ringer's solution. The low values of the ratio, total heat/initial heat, after poisoning with these dithiols indicate that the total heat is little more than the sum of the initial and delayed anaerobic heats.

The lack of reversibility of this action by simple diffusion on soaking repeatedly and for prolonged periods suggests that if the action involves a union between

these substances and oxygen transport enzymes, its dissociation constant is very low.

Various substances known to increase the O_2 consumption of tissues, such as menadione, succinate, fumarate and methylene blue, were employed in an endeavor to prevent the changes caused by BAL, but without success. In these experiments the twitch tension of the double sartorius muscle of the frog in oxygenated Ringer's solution was recorded on smoked paper. Menadione in

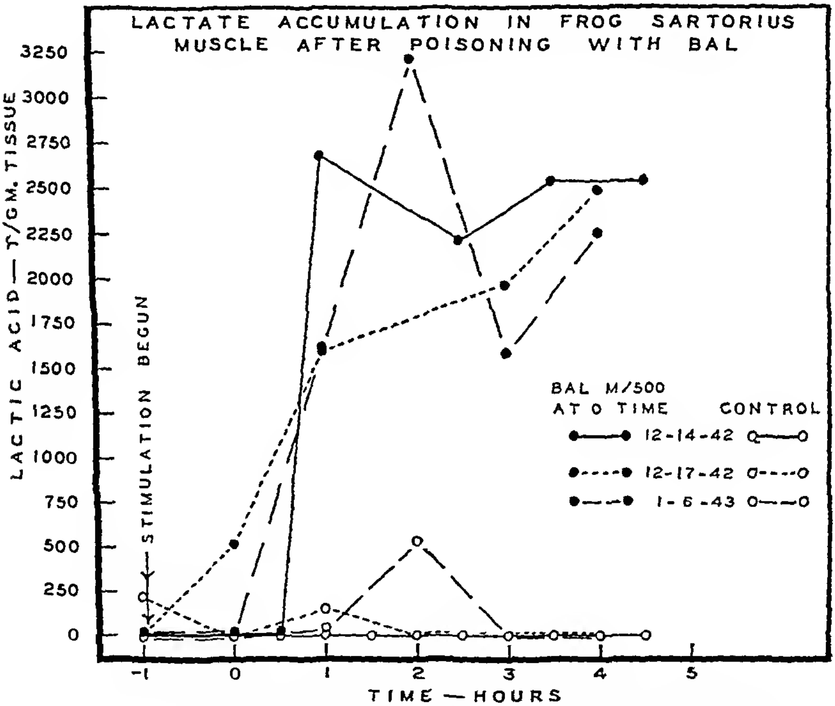

FIG 1 CHANGES IN THE LACTATE CONCENTRATION IN THE LIQUID SURROUNDING STIMULATED SARTORIUS MUSCLES

M/500 BAL was introduced at 0 time in one of each of the three pairs of experiments, the other serving as a control.

0 0001 M concentration did not reverse or prevent the fall in twitch tension resulting from BAL 0.002 M; menadione in higher concentrations, 0.001 M, causes rigor and inexcitability. Similarly, methylene blue in 0.0001 M and 0.0002 M did not prevent the fall in twitch tension after BAL 0 001 and 0.002 M. Methylene blue alone in concentrations of M/1000 or higher was toxic, i.e., caused a fall in twitch tension

The effects of succinate were tested on the inhibition of the oxidation recovery

heat of frog muscle by BAL (see table 3). It will be noted that the results were all negative, including one experiment with menadione and one with fumarate, thus substantiating the results outlined in the preceding section.

TABLE 2

The effect of the ethyl ether of BAL and hexanedithiol-1,6 on the oxidative recovery heat of the isolated frog's sartorius muscle

DATE	DRUG	CONC. MOLAR	OXIDATIVE HEAT IN PER CENT OF INITIAL HEAT			
			Control	After drug	After Ringer's sol.†	
12/16/42	Ethyl ether of BAL	0.001	97	17 (60)*		
12/22/42	Ethyl ether of BAL	0.0005	71	13 (60)		
3/ 8/43	Ethyl ether of BAL	0.0005	78	14 (95)	0 (11¾)	
3/10/43	Ethyl ether of BAL	0.0005	60	21 (60)	15 (1½)	11 (2½)
3/11/43	Ethyl ether of BAL	0.0005	60	10 (60)	6 (1)	5 (2¾)
3/12/43	Hexanedithiol-1,6	0.001	66	39 (60)	0 (2)	
3/16/43	Hexanedithiol-1,6	0.001	98	5 (107)		

* Figures in parentheses indicate time of exposure in minutes.

† Time of soaking in hours in parentheses.

TABLE 3

The influence of succinate, fumarate and menadione on oxidative recovery heat of muscles poisoned with BAL

DATE	OXIDATIVE HEAT,* CONTROL	CONC. ANTAGONIST, MOLAR	OXIDATIVE HEAT*	CONC. BAL, MOLAR	OXIDATIVE HEAT*	CONC. ANTAGONIST, MOLAR	OXIDATIVE HEAT*
5/ 7/43	80	0.0035 succinate	59 (1 hr.)†				
			103 (1¾ hrs.)	0.002	20 (55 min.)	0.0035 succinate	29 (55 min.)
5/14/43	76			0.001	37 (60 min.)	0.005 succinate	31 (60 min.)
5/15/43	90			0.001	28 (45 min.)	0.002 succinate	30 (50 min.)
						0.002 fumarate	31 (45 min.)
4/15/43	52	0.0001 menadione‡	58 (47 min.)	0.001	5 (40 min.)	0.0001 menadione	3 (40 min.)

* In per cent of initial heat.

† Figures in parentheses indicate time of exposure.

‡ Three additional experiments with menadione 0.001 M were inconclusive because it caused an irreversible shortening of the muscle.

DISCUSSION. The experiments confirm the inhibition of oxidation in isolated tissues reported by others. It should be noted that the values for the delayed heat are lower in relation to the initial heat than was the case in the experiments

of Hill (5) and in experiments from this laboratory (6) where the two phases were found to be approximately equal. The control values for the initial heat in the present series of experiments are somewhat variable and average only 62 per cent of the initial heat. The reason for this is not clear, but is probably related to the condition of the frogs which are known to exhibit seasonal variations. For example, it was reported by Barger and Johnson (7) that a considerable proportion of frogs, caught in the spring and kept without food, had gastrocnemi which did not break down glycogen when contracting anaerobically. The fact that the control values were low in the experiments here described does not detract from the significance of the inhibitory action of BAL. The reduction, and in most cases the complete abolition of the oxidative heat, and during the initial phases of poisoning, the augmentation of the twitch tension and initial heat indicate that BAL inhibits oxidative processes and thereby imposes essentially anaerobic conditions upon the muscle. Accumulation of lactate, normally removed by oxidative means, is chemical confirmation of the physical evidence of arrested oxidation. This is also indirect confirmation of the finding that in dogs blood lactate increases following BAL administration and indicates that the increased blood lactate in the intact animal is not a consequence of pulmonary edema or other mechanical factors of respiration, but rather a direct interference with cellular oxidation. Our experiments provide no information on the locus in the oxygen (or hydrogen) transfer chain of reactions at which BAL exerts this inhibitory effect, other than that it apparently does not interfere with the series of reactions leading to the formation of lactic acid. The fact that oxidative recovery processes may be completely inhibited without interference with contractile function (anaerobic) gives to the method a high degree of specificity not shared by other isolated tissue technics.

SUMMARY

In the isolated sartorius muscle of the frog BAL (M/2000 to M/500) caused a marked reduction or complete abolition of the oxidative heat associated with aerobic contraction. The initial heat and twitch tension were at first increased and later reduced, without significant change in "efficiency". Lactate accumulated in the bath fluid. Similar changes were produced by two related dithiols, hexanedithiol-1,6 and the ethyl ether of BAL. Normal function was not restored by menadione, succinate, fumarate or methylene blue.

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THE TREATMENT OF EXPERIMENTAL ARSENIC POISONING WITH THE DITHIOLS[1]

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The interaction of arsenic with the sulfhydryl groups of protein has been well established (1, 2, 3, 4, 5, 6). Since the report of Voegtlin, Dyer, and Leonard in 1923 (1), a number of sulfhydryl compounds have been tried in the treatment of arsenic poisoning, both experimentally and clinically. Glutathione, cysteine, thioglycollic acid, and sodium thiosulfate have all been extensively investigated (7, 8, 9, 10); none has proven an efficient antidote.

In contrast to the above compounds, the dithiols as represented by 2,3-dimercaptopropanol (BAL), exhibit a far greater affinity for arsenic (11). When mixed with either inorganic or organic arsenic, the dithiols form a highly insoluble and stable complex of dithioarsenite (12). It is this characteristic which makes for their efficient antidotal action in arsenic poisoning.

The British investigators early pointed out the effectiveness of topically applied BAL in preventing systemic arsenic poisoning occurring after lewisite contamination (11). As a result of this it became apparent that BAL might be utilized systemically for the treatment of arsenic poisoning in general. This possible application of the dithiols, however, was retarded by their toxicity. The experiments described herein were carried out in 1943 at a time when a thorough study of the pharmacology and toxicology of BAL and related dithiols had provided much needed information (11, 14, 15, 16). At this time Eagle reported that BAL was effective in the treatment of arsenic poisoning in rabbits (17). He found that all rabbits recovered from a lethal dose of phenyl arsenoxide after the administration of five per cent BAL in peanut oil by muscle. He further demonstrated that BAL was capable of increasing the rate and amount of arsenic excretion in these animals.

This report deals with the antidotal efficacy of two dithiol compounds in the treatment of experimental arsenic poisoning in cats. The compounds studied were: BAL (2,3-dimercaptopropanol), and its ethyl ether (2,3-dimercaptopropyl ethyl ether).

Systemic arsenic poisoning was produced in cats by the intravenous injection of mapharsen (phenyl arsenoxide HCl), in a dose of 8 mg. per kg., which was slightly in excess of the LD 100 dose for cats. The dithiol antidote was administered intramuscularly within a few minutes after the arsenic injection.

[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

Table 1 shows the results obtained by BAL treatment of 16 cats acutely poisoned with arsenic. All of these animals were given a single intramuscular njection of two per cent BAL in cottonseed oil. The BAL dosage varied from 0.01 mM per kg. to 0.20 mM per kg. The minimal dose of BAL affording complete protection in these cases was approximately 0.02 mM per kg. The symptoms of arsenic poisoning were negligible in the cats treated with the smaller doses of BAL; they were intensified and severe with the higher doses. Although ultimately providing complete protection, the larger BAL doses failed to alleviate

TABLE 1

Effect of intramuscular BAL in oil on the toxicity of intravenous mapharsen in the cat

NO OF CATS	BAL	PER CENT MORTALITY	SEVERITY OF SYMPTOMS
	mM /kg		
4	0	100	++++
4	0 20	0	++++
2	0.10	0	+++
2	0.05	0	++
2	0 025	0	+
1	0 02	0	+
5	0.01	40	++

Legend· + Depression, nausea
++ Vomiting, salivation, and lacrimation.
+++ Reflex hyperexcitability
++++ Convulsion

TABLE 2

Effect of intramuscular BAL in saline on the toxicity of intravenous mapharsen in the cat

NO OF CATS	BAL	AVERAGE SURVIVAL TIME OF FATALITIES	PER CENT MORTALITY	SEVERITY OF SYMPTOMS
	mM /kg	*min*		
4	0	99	100	++++
6	0.2	24 hrs.	17	++++
4	0 18 (2)*	287	76	++++
2†	0 18 (2)	247	100	++++

Legend: Same as for table 1.
* Figures in parentheses indicate number of such doses given
† These cats were poisoned with 6 mg /kg. of mapharsen.

symptoms and in fact contributed to them. The symptoms of BAL poisoning will be described elsewhere (15), and it need only be noted here that they resemble in many respects those resulting from arsenic.

Table 2 gives the results of experiments on 12 cats treated with an aqueous solution of BAL. A two per cent solution of BAL in saline was given by muscle immediately after the intravenous mapharsen. The doses are indicated in the table. The results in this series were neither as impressive nor as consistent as those obtained by treatment with BAL in an oil medium. The mortality, how-

ever, was reduced to 17 per cent by the single administration of 0.2 mM per kg. of the aqueous BAL. Protection was further evidenced by the longer survival times of the treated animals. In view of the greater toxicity previously noted with the higher BAL doses, it is noteworthy that here the repetition of this relatively large dose served to increase rather than decrease the mortality.

The superiority of the oil solution of BAL is probably due to a slower absorption which in turn would tend to decrease its toxicity and provide a more prolonged action. Furthermore, when BAL is suspended in an aqueous medium, it is less uniform and less stable.

In a few experiments aqueous BAL administered to cats by vein, either before or after intravenous arsenic, failed to prevent a fatal outcome. The symptoms of poisoning were severe and death ensued more rapidly than with the arsenic alone. This is not surprising in view of the evidence that both agents are injurious to the capillaries. On this basis it is concluded that intravenous BAL would be unsuitable for clinical use.

TABLE 3

Effect of intramuscular BAL ether in oil on the toxicity of intravenous mapharsen in the cat

NO OF CATS	BAL ETHER	AVERAGE SURVIVAL TIME OF FATALITIES	PER CENT MORTALITY	SEVERITY OF SYMPTOMS
	mM./kg.	*min.*		
4	0	99	100	++++
7	0.14		0	+
10	0.07	408	40	+
3	0.05	132	34	++
5	0.035	110	60	++
2	0.018	743	100	+++

Legend: Same as for table 1.

These observations were extended to the use of the ethyl ether of BAL in the treatment of mapharsen poisoning in cats. Each of 27 cats received an intravenous injection of 8 mg. per kg. of mapharsen. This was followed promptly by the intramuscular administration of a two per cent solution of the BAL ether dissolved in cottonseed oil. The results are shown in table 3.

The doses employed ranged from 0.018 mM per kg. to 0.14 mM per kg. Protection was apparent with all but the smallest dose. Only the highest dose, however, afforded complete protection. The symptoms of arsenic poisoning were minimal in all cases and significantly less than those seen in the BAL treated animals. The effects observed were blepharospasm, lacrimation, salivation, and signs of nausea. These were in evidence for from six to seven hours with complete recovery by the following day. The more severe symptoms of hyperexcitability and convulsions, which were observed frequently in the BAL treated cats, especially with the larger doses, were never seen in the animals treated with the ethyl ether of BAL.

It is concluded from these experiments that while on a molecular basis the dose

of the BAL ether necessary to save all cats poisoned with mapharsen, was five to seven times that of BAL, the accompanying symptoms were definitely less. It is noteworthy that although the dose of BAL ether was in the range of that which produced chronic toxicity in cats when given alone, such toxicity was never seen in the mapharsen poisoned animals. This suggests a mutual antagonism between these agents.

In 1942 Kensler and Rhoads reported that the inunction of the ethyl ether of BAL was effective in protecting rabbits, dogs, and monkeys against the lethal effects of arsine inhalation (18). These findings were confirmed in the cat in experiments done in collaboration with Mr. Kensler of the Memorial Hospital, New York City.

A total of 13 cats were exposed for a 20 minute period to arsine gas in a chamber

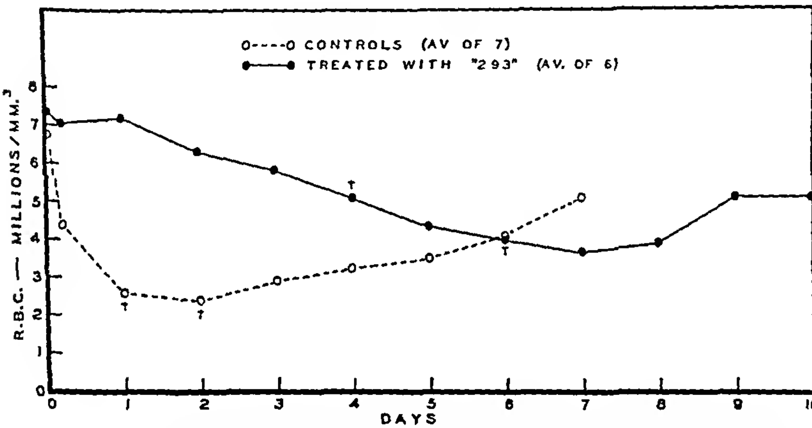

FIG. 1. THE EFFECT OF THE ETHYL ETHER OF BAL ("NDR-293"), ON THE CHANGES IN THE RED BLOOD CELL COUNT OF CATS EXPOSED TO ARSINE GAS
Each cross represents the death of an animal

which provided for a constant circulation of air and gas mixture. The concentration of arsine gas was 0.45 mg. per liter of air. Control hemoglobin determinations and red blood cell counts were made on each animal and repeated daily for at least one week. Seven of the animals were untreated after their removal from the chamber; the remaining six were treated with the ethyl ether of BAL applied to the skin.

The results are shown in figure 1. In the untreated group five cats survived during an observation period of at least one week; two died within 24 hours after exposure. In the control animals a gross hemoglobinuria developed, and the average red cell count fell 63 per cent in 24 hours; the average maximum reduction for the period was 75 per cent. The changes in the hemoglobin concentration, (not shown in the figure), paralleled those of the red blood cell counts but were of less degree.

In the experimental group three cats were treated immediately after their removal from the chamber by the percutaneous application of the ethyl ether of BAL in a dose of 0.28, 0.35, and 0.41 mM respectively. The same dose was repeated four hours later. These animals all survived and hemoglobinuria was not manifest. There was no fall in the red cell count or hemoglobin level until the third day in two of the cats and not until the fifth day in the other.

The remaining three cats were treated in similar fashion with the exception that treatment was withheld until one hour after their removal from the chamber. The dosage of the BAL ether was increased to 0.41, 0.48, and 0.55 mM respectively in these cases. The two smaller doses were repeated after a four hour interval. The cat that received the 0.55 mM dose manifested signs of dithiol toxicity and further treatment was not given. This animal died seven days later of inanition characteristic of chronic poisoning produced by this dithiol. The cat treated with the 0.48 mM dose died six days later of an upper respiratory infection and pneumonia. The other animal survived and was entirely normal at the end of two weeks. Hemoglobinuria did not appear in these animals and there was no immediate fall in either the red cell count or hemoglobin level. As may be seen from the chart, hemolysis was largely inhibited and much more gradual than was the case in the controls. Similar results were obtained in three cats poisoned with arsine gas and treated with a single intramuscular injection of the BAL ether in cottonseed oil.

In view of the striking protection afforded by the dithiols against arsenic poisoning, it became of interest to determine the relative toxicity of the dithioarsenite complex formed in the course of the antagonism. The BAL dithioarsenite was prepared in vitro and administered to three cats intravenously in doses of 0.02 mM, 0.06 mM, and 0.08 mM per kg. respectively. The doses represent one, three, and four times the LD 100 vein dose of mapharsen for the cat. All of the animals survived and symptoms of poisoning were manifest only in the cat receiving the highest dose. From this it is evident that the dithioarsenite is relatively non-toxic in comparison to either mapharsen or BAL.

The results of the experiments described in this report indicate the value and specificity of these dithiols in the treatment of acute arsenical poisoning.

SUMMARY

1. BAL given by muscle in cottonseed oil in suitable dosage provides complete protection to cats acutely poisoned with mapharsen.

2. With higher doses of BAL protection is complete but signs of poisoning are intensified.

3. BAL in oil is superior to BAL in saline as an antidote in mapharsen poisoning.

4. The minimum effective dose of the ethyl ether of BAL providing complete protection to cats poisoned with mapharsen, is five to seven times that of BAL.

5. Signs of poisoning were minimal in cats treated with the BAL ether and significantly less than those occurring in the BAL treated animals.

6. The ethyl ether of BAL protects cats against the lethal action of arsine gas, and effectively diminishes the associated hemolytic effects.

7. The intravenous administration of the dithioarsenite of BAL is relatively non-toxic to cats.

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THE EFFECT OF 2,3-DIMERCAPTOPROPANOL (BAL) ON THE WHOLE BLOOD AND PLASMA CONCENTRATION OF ARSENIC AFTER MAPHARSEN IN CATS[1]

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The dithiol, 2,3-dimercaptopropanol (BAL), has been shown to be effective in the treatment of arsenic poisoning in cats, rabbits, and man (1, 2). Eagle has shown that the urinary excretion of arsenic in poisoned rabbits is greatly increased after the intramuscular administration of 5 per cent BAL in peanut oil (1). In a study carried out by the Cooperative Clinical Group it was found that the intramuscular administration of BAL in peanut oil to patients with various forms of arsenical poisoning produced a definite clinical improvement in the majority in a period shorter than usual, and facilitated the urinary excretion of arsenic (1). As a result of these studies it appears that BAL is capable of mobilizing tissue bound arsenic thereby promoting its excretion.

The experiments described in this report were carried out in order to determine whether BAL actually effects a shift of arsenic from tissues to blood as reflected by the blood arsenic concentration.

METHODS. The experiments were carried out on 16 cats. Five animals were given a single intravenous dose of 3 mg. per kg. of mapharsen; whole blood and plasma arsenic levels were determined one minute after injection, and at hourly intervals therafter for as long as 6 hours. In one case a sample was drawn at 30 minutes and in another at 22 hours. Six cats receiving a similar dose of mapharsen were given a subsequent intramuscular dose of 0.02 mM per kg. of BAL dissolved in cottonseed oil (1:50). The interval between the mapharsen administration and the BAL treatment was varied. In 3 of the 6 cats the BAL was administered 4 hours after the mapharsen and in the remaining 3 the BAL was injected after an interval of 18 hours. In all cases a blood sample for arsenic determination was drawn just prior to the BAL injection.

The arsenic determinations were performed according to the method of Chaney and Magnuson (3).

RESULTS. The curve of the elimination of arsenic from the blood stream was plotted from the results obtained on 5 cats given a single intravenous dose of mapharsen. The results are shown in figure 1. The curve of elimination shows 3 phases. First there is a rapid fall in the arsenic concentration of both the whole blood and the plasma. Within less than 60 minutes there is a levelling off after which the curves decline gradually and diverge. The plasma arsenic falls more rapidly and reaches zero in 5 to 6 hours. The whole blood arsenic level is main-

[1] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

[2] This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the names of the chief collaborators.

tained at a relatively high level for at least 6 hours and possibly 22 hours, as found in one cat tested at this time.

Within one minute after the injection the whole blood arsenic represented an average of about one-half of the amount injected; this was calculated on the basis of the blood volume being equal to 10 per cent of the body weight. At the end of 1 hour the blood contained 10 per cent of the injected dose. This is consistent with the assumption that at this time the arsenic is equally distributed between the blood and the rest of the tissues. However, the liklihood is that distribution takes place much faster; in one case in which a blood sample was analyzed 30 minutes after the mapharsen injection the blood was calculated to contain 10 per cent of the total dose.

The curves indicate clearly that arsenic is fixed in the cells, since the concentration in the whole blood is well sustained in contrast to its rapid disappearance from the plasma. The calculated arsenic content of the red cells based on an average hematocrit value of 40, is indicated in figure 1.

The effect of BAL on the concentration of arsenic in the whole blood and plasma, studied in six cats is shown in figure 2. The results are uniform in demonstrating that after BAL treatment the concentration of arsenic in the whole blood and plasma is increased. Following the BAL injection there is a sharp rise in the arsenic content of the whole blood and plasma during the first hour. The whole blood arsenic level continues to rise more slowly during the second hour, and then declines rapidly, reaching a concentration approximately that of the control level in 4 hours.

The effect of BAL on the plasma arsenic concentration can be seen from the figure to parallel the results obtained with the whole blood. However, if it is assumed that the increase in whole blood arsenic concentration is solely in the plasma fraction then the concentrations in the plasma should be 2 to 3 times those found. On the basis of the values obtained for plasma arsenic concentration, it would appear that the concentration of arsenic in the red cell is increased following BAL. This seemed improbable and suggested the possibility that the concentrations of arsenic found might not be truly representative of that in the plasma. The possibility was considered that the insoluble thio-arsenite of BAL might be partially thrown down on centrifugation and thus give rise to a lower plasma arsenic concentration than actually exists. Direct evidence supporting this view was obtained by the following experiments carried out on blood samples from 2 cats which had previously received mapharsen and BAL. Each sample was divided into two parts, one of which was allowed to clot, and the other citrated and centrifuged. The concentration of arsenic in the serum (not centrifuged) was found to be approximately 3 times that of the plasma (centrifuged). In another similar experiment the suspension of citrated red cells obtained by centrifugation and the precipitate at the bottom of the tube were analyzed separately. About 55 per cent of the total arsenic contained in the suspension was recovered from the portion at the bottom layer which represented only 4 per cent of the total volume. It is evident from this that the increase in

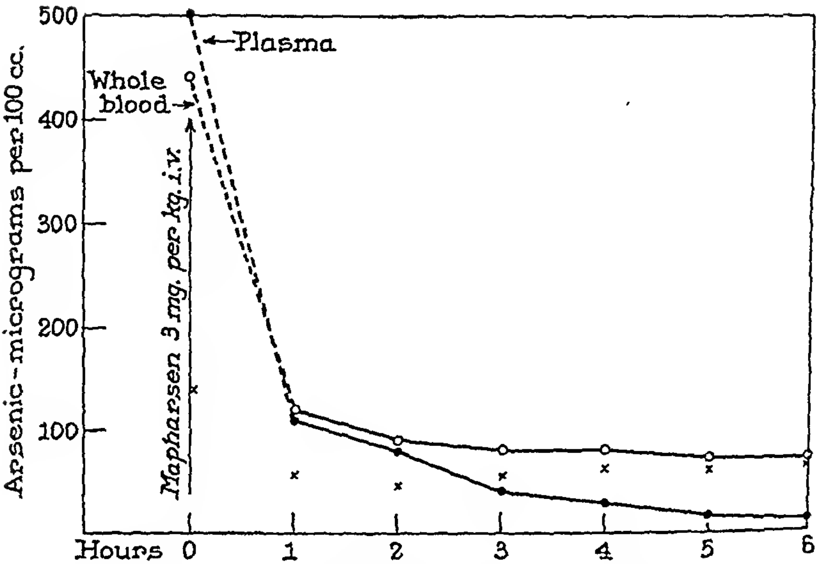

Fig. 1. Curve of the Elimination of Arsenic from the Blood Stream. Average of 5 Experiments

The crosses represent the calculated values of red cell arsenic concentration.

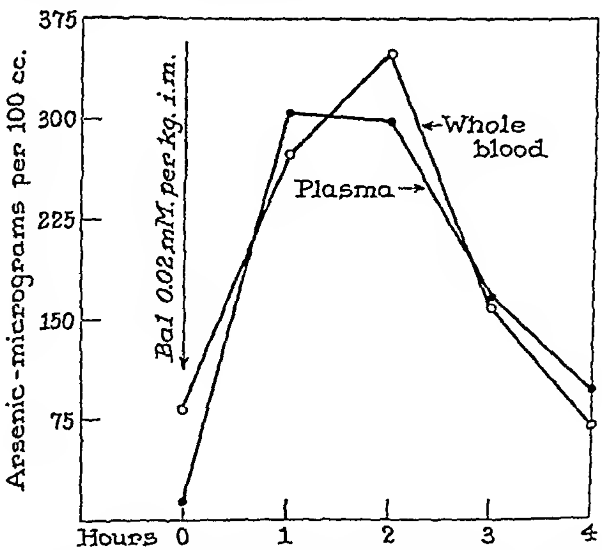

Fig. 2. The Effect of BAL on the Concentration of Arsenic in the Whole Blood and Plasma. Average of 6 Experiments

whole blood arsenic concentration following BAL is essentially in the plasma fraction.

The results are in accord with the concept that BAL effects a withdrawal of arsenic from the tissues. The elimination of the thio-arsenite complex from the blood probably takes place via the kidneys as has been reported for rabbits and man. Eagle has shown that the peak excretion of arsenic in the urine of rabbits poisoned with mapharsen and treated with BAL, occurs in from 2 to 4 hours after the injection of BAL (1). This agrees well with the time at which the maximal blood concentrations appeared in the cats, since a high level of arsenic was attained within 1 hour after BAL and the peak was reached by the second hour.

The dose of BAL administered to the cats in these experiments represented 50 per cent more BAL than was necessary to combine with the entire mapharsen dose on a mole per mole basis. It seemed desirable therefore to determine whether a second dose of BAL could mobilize additional arsenic and reestablish a high level in the blood. Mapharsen was administered in doses of 3 mg. per kg. intravenously to each of 5 cats. A dose of BAL, 0.02 mM per kg., was given intramuscularly 4 hours later. This dose of BAL was repeated again 4 hours after the first in 3 animals and 24 hours after the first in the remaining 2. A control sample of blood was taken prior to the second BAL injection and samples were drawn at hourly intervals thereafter. The arsenic concentration of the whole blood was determined. The results were uniform in showing that the second dose of BAL was without effect on the concentration of arsenic in the whole blood.

These results indicate that the first dose of BAL was sufficient to mobilize all of the available arsenic. From the therapeutic standpoint the failure to obtain a rise in the blood level of arsenic after a dose of BAL may serve as a guide to the inference that previous doses of BAL have been sufficient.

SUMMARY

1. The curve of the elimination of arsenic from the blood stream was determined on 5 cats given a single intravenous dose of mapharsen.

2. The plasma arsenic falls to 0 in 5 to 6 hours; the whole blood arsenic is maintained at least 6 hours and possibly 22 hours, indicating clearly the fixation of arsenic by the red cells.

3. Six cats similarly poisoned were given a single intramuscular dose of BAL in oil, either 4 or 18 hours after the arsenical.

4. The BAL dose effected a sharp rise in the whole blood and plasma arsenic concentrations. The peak effect was reached in 2 hours.

5. The increased blood arsenic concentrations following BAL is essentially in the plasma fraction in accord with the concept that BAL effects a mobilization of tissue arsenic.

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POISONING FROM INHALATION OF THE VAPORS OF LEWISITE AND PHENYLDICHLORARSINE: ITS PATHOLOGY IN THE DOG AND TREATMENT WITH 2,3-DIMERCAPTOPROPANOL (BAL)[1]

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In view of the effectiveness of 2,3-dimercaptopropanol in the prophylaxis and therapy of the systemic toxic effects of lewisite and phenyldichlorarsine following their application to the skin (1), the following series of experiments was undertaken to test its value in the treatment of dogs exposed to the vapors of these chemical warfare agents. Both of these compounds have an exceedingly low volatility and hence it is unlikely that lethal concentrations of their vapors would be attained under combat conditions. The significance of the present study, in which 2,3-dimercaptopropanol was found to be efficacious, is, therefore, largely theoretical.

EXPERIMENTAL TECHNIQUES. Lewisite (chlorvinyldichlorarsine) or phenyldichlorarsine, atomized in a nebulizer described and provided by the University of Chicago Toxicity Laboratory (2), was introduced at a constant rate into the intake of an 850 liter chamber through which air was drawn at a rate of 900 liters per minute. The nominal concentration of the vapor could be calculated from values for the duration of exposure, velocity of air flow, and loss in weight of the contents of the nebulizer during the exposure. The actual concentration was determined in each instance by analysis of the chamber atmosphere after equilibrium had been attained, i.e., 4 minutes after beginning the exposure. A measured volume of the chamber atmosphere was drawn through 2 absorption towers in series, each containing a 5% solution of sodium hydroxide in 50% ethyl alcohol. (Aqueous NaOH was satisfactory for the absorption of lewisite.) The towers were placed in the chamber close to the cage containing the experimental animal. Their pooled contents were analyzed for arsenite by standard iodimetric procedure and for chloride by Volhard titration.

There was considerable variation in the volume of arsenical atomized in successive exposures, and even greater variability was encountered in the actual concentrations realized (tables 1, 2, and 3). Concentrations determined by analysis for chlorine, not here tabulated, were on the average 20% higher than those based on analysis for arsenic. This discrepancy, as well as the marked disparity between nominal and analytical concentrations, has been observed by Hutchens and Hein (3) and can be explained by the rapid hydrolysis of the vaporized arsenical to an arsenoxide and hydrochloric acid; the former is largely adsorbed on the surfaces of chamber and cage and on the fur of the experimental animal, while some of the latter remains free in the chamber atmosphere.

Dogs were gassed singly; equal numbers of treated and control animals were exposed concurrently. The flow of air and gas was started after the dog had been placed in the chamber. Gas was introduced for a period of 30 minutes in most instances. The circulation of air was continued for 10 minutes after the gas was turned off, and the animal removed at this time.

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Yale University.

At the beginning of the study, treatment was by the intravenous route. 2,3-dimercaptopropanol was administered as a 0.25% solution (0.02M) in physiological saline; 7 equal injections of 5 mgm. per kilogram of body weight were given at intervals of 15 minutes,

TABLE 1

Effect of 2,3-dimercaptopropanol administered intravenously in a dosage of 35 mgm. per kilo on the mortality of dogs exposed to lewisite vapor

TREATMENT STARTED AT	AVE. CONC. OF TOXIC VAPOR (MGM./L.) ± S.D.		ACUTE DEATHS (NUMBER OF DOGS)				LATE DEATHS 2-7 days	TOTAL MORTALITY
	Nominal	By As. analysis	0-6 hrs.	6-12 hrs.	12-24 hrs.	24-48 hrs.		
A. 30 minute exposure								
Control........	0.139 ± 0.025	0.050 ± 0.013	2	4	7	5	4	22/27
Immediately...	0.131 ± 0.018	0.045 ± 0.010	0	0	1	0	3*	4/10*
30 minutes.....	0.144 ± 0.018	0.053 ± 0.014	0	0	0	0	0	0/8
90 minutes.....	0.148 ± 0.008	0.053 ± 0.010	0	0	2	1	0	3/8
B. 10 minute exposure								
Control........	0.384 ± 0.048	0.144 ± 0.015	1	0	1	1	1	4/6
30 minutes.....	0.351 ± 0.005	0.121 ± 0.015	0	0	1	0	0	1/5

* One dog with extensive pneumonia sacrificed at 5 days, listed as a fatality.

TABLE 2

Effect of 2,3-dimercaptopropanol administered intramuscularly in a dosage of 20 mgm. per kilo on the mortality of dogs exposed to lewisite vapor for 30 minutes

TREATMENT STARTED AT	AVE. CONC. OF TOXIC VAPOR (MGM./L.) ± S.D.		ACUTE DEATHS (NUMBER OF DOGS)				LATE DEATHS, 2-7 DAYS	TOTAL MORTALITY
	Nominal	By As. analysis	0-6 hrs.	6-12 hrs.	12-24 hrs.	24-48 hrs.		
Control........	0.143 ± 0.006	0.065 ± 0.009	1	3	2	0	2	8/10
30 minutes....	0.143 ± 0.003	0.061 ± 0.007	0	0	4	0	2	6/9

TABLE 3

Effect of 2,3-dimercaptopropanol administered intramuscularly in a dosage of 40 mgm. per kilo on the mortality of dogs exposed to phenyldichlorarsine vapor for 30 minutes

TREATMENT STARTED AT	AVE. CONC. OF TOXIC VAPOR (MGM./L.) ± S.D.		ACUTE DEATHS (NUMBER OF DOGS)				LATE DEATHS, 2-7 DAYS	TOTAL MORTALITY
	Nominal	By As. analysis	0-6 hrs.	6-12 hrs.	12-24 hrs.	24-48 hrs.		
Control ...	0.159 ± 0.006	0.062 ± 0.012	2	2	3	2	0	9/11
30 minutes	0.160 ± 0.006	0.055 ± 0.009	0	0	2	1	1	4/11

totaling 35 mgm. per kilo over a period of 1½ hours. In 3 groups of dogs that had been exposed to lewisite vapor for 30 minutes, treatment was commenced immediately, 30 minutes, and 90 minutes respectively after removal of the animal from the chamber (i.e., 40,

70, and 130 minutes after beginning the exposure) (table 1A). In a fourth group, exposed for 10 minutes to a higher concentration of the gas, treatment was begun 30 minutes after removal of the animal (50 minutes after beginning the exposure) (table 1B).

For the remainder of the study a 5 or 10% solution of BAL in peanut oil with 10 or 20% benzyl benzoate respectively was used.[2] A single injection of 20 mgm. per kilo was given intramuscularly to a group of dogs exposed to lewisite vapor, 30 minutes after a 30 minute exposure (70 minutes after beginning the exposure) (table 2). The same amount of BAL was given at the same post-gassing interval to a group of dogs following a 30 minute exposure to phenyldichlorarsine vapor, and repeated 2 hours later for a total dosage of 40 mgm. per kilo (table 3).

Surviving animals were sacrificed 7 or more days after gassing. Gross inspection of the larynx, trachea, and lungs of all animals was made, and sections were taken for histological examination in selected cases.

Results of Experiments. Inasmuch as the toxic effects of both gases were indistinguishable and the response of dogs to treatment with BAL was equally good after gassing with either lewisite or phenyldichlorarsine, the results of experiments with both arsenicals may be combined in a single discussion.

Clinical course. Great excitement and overactivity were apparent as soon as gassing commenced and continued for a variable period of time, up to the duration of the exposure. Retching, vomiting, urination, and defecation were customarily observed. Respiratory distress and marked salivation were noted when the animals were taken out of the chamber. Respiratory obstruction was the cause of death in those dogs dying acutely, i.e. within 48 hours. Animals that died later, and a few surviving dogs as well, developed signs of pneumonic consolidation by physical and roentgenographic examination. In a few cases there was a purulent conjunctivitis.

Mortality (tables 1, 2, and 3). Approximately 80% of the dogs in each control group died. One fourth to one half of the fatalities in the untreated animals occurred within 12 hours. The earliest death was at 3 hours. There was a marked reduction in mortality following the administration of BAL in a dosage of 35 or 40 mgm. per kilo, even when the initiation of treatment was delayed until 90 minutes following gassing. BAL effected no significant change in mortality when administered in a dosage of 20 mgm. per kilo 30 minutes after gassing. None of the treated dogs, even in the group receiving inadequate dosage, died within 12 hours after exposure. There were too few dogs gassed with lewisite for 10 minutes to lend statistical significance to the difference in mortality between treated and untreated animals in this group.

Pathology. Acute deaths, occurring within 48 hours, were attributable to an ulcerating, necrotizing laryngotracheobronchitis with resultant respiratory obstruction from a thick pseudomembrane of fibrin, necrotic epithelium, and polymorphonuclear leukocytes, extending from the edematous glottis into the small bronchial radicles. The bronchioles were filled with exudate but their mucosa was less severely affected. There was marked edema of the peribronchial, perivascular, and mediastinal tissues and of the alveoli, which were the site of hemorrhage and occasionally emphysema. Penumonia in association

[2] The BAL in peanut oil was furnished by Surgeon Harry Eagle, U. S. P. H. S.

with the acute bronchiolitis was present in those animals dying 2 days after gassing.

With but one exception, all the late fatalities (those dying 2 to 7 days after exposure) were due to progression of the laryngotracheobronchitis with a complicating pneumonia. Frankly purulent foci were cultured at postmortem; Staphylococcus albus and aureus were ordinarily recovered, and in one instance Streptococcus viridans. In the later fatalities of this group there was regeneration of a metaplastic stratified squamous epithelium in the bronchi.

In about 50% of the survivors sacrificed 7 to 12 days after gassing, no pulmonary lesions were found on gross examination, though the lungs were somewhat heavier than normal and there was a lack of their normal tendency to collapse. In the remaining animals, small zones of edema, emphysema, atelectasis, and consolidation were seen. In all surviving dogs, histological evidence of previous damage to the trachea and bronchi was found in epithelial metaplasia and slight submucosal edema and inflammatory exudation. Peribronchiolar fibroblastic proliferation and focal pneumonia, usually not extensive, were not infrequently observed.

One treated dog that was sacrificed 3 months after exposure to phenyldichlorarsine showed slight thickening of the alveolar walls.

DISCUSSION. 2,3-dimercaptopropanol was found to be effective in treatment of poisoning by inhaled lewisite and phenyldichlorarsine vapor; the dosage required in successful treatment was well below the LD_{50} for the dog: 35 mgm. per kilo intravenously administered over a period of 90 minutes or 40 mgm. per kilo intramuscularly in 2 hours in contrast to the LD_{50} which is 100 mgm. per kilo given at one time (4). A smaller dose, 20 mgm. per kilo, was ineffectual. The amounts required are considerably in excess of those which have been administered to humans. Modell, Gold, and Cattell (4) and Sulzberger, Baer, and Kanof, (5) have given 3–4 injections at 4 hourly intervals of the 10% peanut oil preparation intramuscularly into humans. Local discomfort and mild symptoms of systemic toxicity resulted.

In this study it has been demonstrated that BAL is equally effective whether administered intravenously or intramuscularly. This is confirmatory of the studies of its toxicity and of its efficacy in treatment of the systemic action of lewisite burns (6, 1).

SUMMARY

1. Dogs exposed to lethal concentrations of the vapors of lewisite or phenyldichlorarsine develop a necrotizing pseudomembranous laryngotracheobronchitis. Death is usually due to respiratory obstruction occurring within 48 hours. Later deaths are due to a progression of the acute lesions and pneumonia.

2. BAL in a dosage of 35–40 mgm. per kilogram of body weight, administered intravenously or intramuscularly in divided doses, markedly reduced the mortality of dogs exposed to an LC_{50} of the vapors of either lewisite or phenyldichlorarsine, even when treatment was initiated as late as 100 minutes after a 30 minute exposure to the gas.

The authors acknowledge with thanks the technical assistance of Miss Helen Criscuolo.

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THE TREATMENT BY 2, 3-DIMERCAPTOPROPANOL (BAL) OF THE SYSTEMIC TOXIC EFFECTS OF SKIN CONTAMINATION WITH LEWISITE AND PHENYLDICHLORARSINE[1]

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BAL has a twofold action in the treatment of the injury produced by contamination of the skin with liquid lewisite or phenyldichlorarsine. If applied early to the local lesion it can inhibit the necrotizing action of these vesicants and prevent the absorption of the hydrolyzed arsenicals from the local site; if sufficiently large amounts of BAL are applied to either injured or intact skin or if solutions of BAL are injected, concentrations of BAL may be maintained in the body fluids which are effective in preventing the systemic toxic effects of the absorbed lewisite and phenyldichlorarsine oxides.

It has been repeatedly demonstrated in experimental animals that lethal amounts of the arsenical vesicants can be absorbed from a relatively small area of skin contamination (1). The possibility that men splashed with these vesicants might succumb to the toxic effects of the absorbed arsenic compounds prompted the investigation of the most suitable methods of using BAL in the treatment of such casualties.

The absorption of BAL after skin application is not sufficiently rapid to afford maximum protection from the absorbed arsenicals even if undiluted BAL is used. The present studies indicate that the most effective method of treating the systemic effects of skin burns produced by either lewisite or phenyldichlorarsine is by the combined application of BAL to the burned area and the injections of solutions of BAL. The local treatment prevents continued absorption of the arsenicals from the burned area and the systemic treatment is necessary to produce concentrations of BAL in the body fluids which can inhibit the toxic action of the arsenic compounds in the tissues. BAL is itself a quite toxic compound so that the dosages must be carefully regulated to avoid the injurious effects of excess BAL. Efforts to develop a method which could be used for the determination of the concentration of reduced BAL in the blood plasma after the injection of therapeutic doses were unsuccessful.

As the result of preliminary experiments on mice, tentative conclusions were drawn as to the length of time an effective concentration of BAL might persist after its injection. An amount of lewisite equivalent to $3LD_{50}$ (60 mgm. per kg.) was applied to the clipped skin of mice and 30 minutes later the mice were treated either by the application of undiluted BAL to the burned area or by the repeated

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subcutaneous injection of dilute solutions of BAL in saline. The results are shown in table 1 and indicate that the treatment by repeated subcutaneous injections of BAL is more effective than local application if the injections are properly spaced. Treatment must be repeated at approximately 3 to 4 hour intervals for about 12 hours after the lewisite application. The estimate that most of the injected BAL is destroyed or excreted in about 4 hours is in agreement with studies in man which show that there is no appreciable cumulative toxicity of small doses of BAL if injections are spaced at 4 hour intervals (2). The total amount of BAL needed for optimal treatment under those conditions was about 1 mM per kg. and the amount of lewisite applied to the skin was approximately

TABLE 1

Treatment by BAL of systemic toxicity of lewisite burns in mice

METHOD OF TREATMENT	TOTAL AMOUNT OF BAL ADMINISTERED	MORTALITY
	mM/kg.	
Percutaneous*	1.25	10/10
	2.5	9/20
	5.0	22/50
	7.5	5/10
	10.0	9/10
Subcutaneous—3 injections†	0.72	7/10
	0.96	7/10
	1.20	7/10
Subcutaneous—4 injections‡	0.64	5/10
	0.96	2/10
	1.28	1/10
	1.60	8/20

All mice were burned with 60 mgm. (0.3 mM) of lewisite per kg. 30 minutes before treatment was started.

* Undiluted BAL applied to skin including area of burn.

† Total dose divided into 3 equal parts given ½, 5½ and 12 hours after lewisite application.

‡ Total dose divided into 4 equal parts given ½, 3½, 7½ and 12 hours after lewisite.

0.3 mM per kg. The lewisite was probably not completely absorbed from the local area but the results indicate that in the mice an excess of BAL in a ratio of at least 3 mols of BAL to 1 of lewisite is needed for inhibition of the toxic action of the absorbed lewisite oxide.

Repeated subcutaneous injection of solutions of BAL in saline were also given to dogs which had been burned with $2LD_{50}$ of lewisite (38 mgm./kg.). It was impossible to protect effectively these animals from the lethal effects of this dose of lewisite even when a total of 1.8 mM per kg. of BAL was given divided into 5 injections spaced at 4 to 5 hour intervals and the treatment begun simultaneously with the lewisite application. Of 8 dogs so treated only 2 survived but the course of the 6 dogs which succumbed was considerably modified from that seen in untreated animals. In the untreated dog burned with this dose of

lewisite the predominant feature is the rapid onset of pulmonary edema which may prove fatal within 3 hours. None of the animals which were given the treat-

TABLE 2

Treatment by BAL of lewisite poisoning in dogs

(Figures in body of table are mortality rates)

METHOD OF TREATMENT	TIME INTERVAL BEFORE START OF TREATMENT		
	30 min.	60 min.	120 min.
Dogs burned with 2LD$_{50}$ of lewisite (38 mgm./kg.)			
Undiluted BAL, 0.3 cc. per kg., locally	3/10	7/10	
Undiluted BAL locally plus BAL solution subcutaneously, 0.3 mM per kg.	0/10	1/12	4/8
Undiluted BAL locally, 0.1 cc. per kg., plus BAL solution intravenously, 0.04 mM per kg. × 7, given at 15 minute intervals . .		0/8	
5% BAL ointment locally, 2 cc. per kg. .	7/8	8/8	
5% BAL ointment locally plus 10% BAL in oil intramuscularly, 5 mgm. per kg... .. .	5/12		
5% BAL ointment locally plus 10% BAL in oil intramuscularly 5 mgm. per kg. × 2 at ½ and 3½ hours	2/10		
5% BAL ointment locally plus 10% BAL in oil intramuscularly 10 mgm. per kg.	1/8	9/12	
5% BAL ointment locally plus 10% BAL in oil intramuscularly, 10 mgm. per kg. × 2 at 1 and 4 hours.		4/10	
5% BAL ointment locally plus 10% BAL in oil intramuscularly, 10 mgm. per kg. × 3 at 1, 4 and 7 hours.		2/8	
Dogs burned with 4LD$_{50}$ of lewisite (76 mgm./kg.)			
Undiluted BAL locally, 0.5 cc. per kg. . . .	5/10		
Undiluted BAL locally, 0.1 cc. per kg. plus BAL solution intravenously, 0.04 mM per kg. × 7, given at 15 minute intervals.	0/4	10/11	

TABLE 3

*Treatment by BAL of phenyldichlorarsine poisoning in dogs**

METHOD OF TREATMENT†	MORTALITY
None.	12/12
5% BAL locally	5/8
5% BAL locally plus 10% BAL in oil intramuscularly, 5 mgm. per kg. × 2 at 1 and 4 hours	1/8

* Dogs burned with 82 mgm. per kg. of phenyldichlorarsine applied to clipped skin.
† Treatment started 60 minutes after application of phenyldichlorarsine.

ment with BAL described above showed evidence of pulmonary edema and the deaths occurred 24 to 48 hours after the lewisite burn after the dogs had lapsed

into a state of severe shock as manifested by marked drop in blood pressure, tachycardia and loss of plasma volume.

It was evident that for adequate treatment of lewisite burns in dogs the continuted absorption of the lewisite oxide from the local area must be prevented. This can be accomplished by the local application of BAL either undiluted or in an ointment base. The results of the combination of local application of BAL plus the injections of solutions of BAL are shown in table 2. The application of undiluted BAL to the local lesion plus the subcutaneous or prolonged intravenous injection of approximately 0.3 mM (36 mgm.) of BAL per kg. as a dilute solution in saline was very effective. The saline solutions of BAL are unstable and must be prepared immediately before injection. For this reason treatment with a more stable type of preparation of BAL, e.g. a 5 or 10% solution of BAL in peanut oil and benzyl benzoate as devised by Eagle (3, 4), was tried. In place of the local application of undiluted BAL a 5% solution of BAL in an ointment base was used. The BAL ointment was found satisfactorily to inactivate the lewisite remaining at the skin burn even though the absorption of BAL when it is applied in this form is too slow to afford any protection against the systemic toxic effects of lewisite. When the local use of BAL ointment is supplemented by injection of solutions of BAL good therapeutic results are obtained. The effectiveness of therapy is inversely related to the time elapsing between the application of lewisite to the skin and the onset of treatment. Relatively large doses of BAL could be given without toxic symptoms if given in divided amounts at intervals of approximately 3 hours.

A similar form of therapy has been found to be successful in the treatment of dogs burned with a lethal dose of phenyldichlorarsine (table 3). Because the absorption of this compound from the skin of dogs is slower than that of lewisite, less BAL is required for the adequate treatment of the systemic toxicity of phenyldichlorarsine burns.

SUMMARY

The injection of solutions of BAL in saline or oil is of value in the treatment of the systemic toxic effects of lewisite or phenyldichlorarsine following the contamination of the skin with these compounds.

In dogs burned with $2LD_{50}$ or more of lewisite or phenyldichlorarsine the most effective treatment is combination of the application of BAL to the local lesions to prevent further absorption of the arsenical compounds with the injection of solutions of BAL to maintain concentrations of BAL in the tissues which can inhibit the toxic effects of the absorbed lewisite and phenyldichlorarsine oxides.

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THE TREATMENT OF ACUTE CADMIUM INTOXICATION IN RABBITS WITH 2,3-DIMERCAPTOPROPANOL (BAL) AND OTHER MERCAPTANS

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The historical background which led to the synthesis of BAL (2,3-dimercaptopropanol) and the basic contributions which have resulted from studies on the effect of mercaptans on the toxic action of arsenic and other heavy metals have been summarized by Peters (1) and Waters and Stock (2). Among the many fruitful results of recent investigations on the thiol antagonism of heavy metal poisoning is the accumulation of evidence supporting the hypothesis that heavy metals are toxic to biological systems because of their reversible mercaptide-formation with SH groups of the protein moiety of cellular enzymes. As an extension of this hypothesis a reasonable explanation of the mechanism by which dithiols can reactivate poisoned enzyme systems or exert therapeutic benefit in the treatment of heavy metal poisoning is the formation of mercaptides of sufficiently low dissociability to reverse effectively the combination of heavy metals with sensitive cellular enzyme systems.

In the treatment of arsenical poisoning this concept has received support from the demonstration that BAL reacts with lewisite to form a stable mercaptide and that BAL effectively reverses the *in vitro* inhibition of pyruvic acid oxidase caused by arsenicals (1). The effectiveness of BAL therapy in the treatment of systemic intoxication from a variety of toxic arsenicals, both in experimental animals (1, 2, 3) and in man (1, 2, 4), attests to the facility with which the dithiol forms stable thio-arsenites *in vivo*. Likewise, the observation that the inhibition of SH-containing enzymes by mercury can be reversed by BAL (5, 6) has its clinical counterpart in the efficacy of BAL in the treatment of experimental (7) and human (8) mercury poisoning.

The generalization that heavy metals have a common basic mechanism of action and as a group can be antagonized by dithiols receives further support from studies on the actions of cadmium *in vitro* and *in vivo*. It has been shown that cadmium inhibits succinoxidase and that this reaction can be reversed by BAL (5, 6). The following study is concerned with the effects of BAL and related mono- and dithiols on systemic cadmium poisoning. Although systemic cadmium poisoning is an uncommon clinical occurrence, nevertheless, the actions of this metal afford, more than do those of arsenic and mercury, an experimental tool for elucidating the mechanisms by which mercaptans reverse the toxic effects of heavy metals *in vivo*.

[1] Major, Sn-C, A U S.
[2] 1st. Lt, Sn-C, A U S

THE SCREENING OF MERCAPTANS FOR THEIR EFFICACY IN THE TREATMENT OF SYSTEMIC CADMIUM POISONING. Forty-four mercaptans have been screened to determine their efficacy in the treatment of systemic cadmium poisoning. Rabbits were used as experimental subjects. The lipoid soluble mercaptans were injected in a solution of propylene glycol containing varying amounts of alcohol to effect solution. They were given in 3 equally divided doses totalling 0.3 mM/Kg., the first intravenously, either one minute before or 30 minutes after the intravenous injection of cadmium, and the second and third, intramuscularly, 1 and 3 hours later, respectively. Cadmium was administered as a solution of its chloride salt, the standard dose employed being 5.0 mg./Kg. The mercaptans studied were, for the most part, dithiols of aliphatic hydrocarbons ranging from dithio-ethane to dithiohexadecane. Also included were various corresponding alcohols, acids, ethers and esters. The SH groups were variously placed in the molecule. As a group the dithiols were uniformly inferior to BAL in counteracting the effects of systemic cadmium intoxication. Many were highly toxic in the dose employed. Suffice it to say that only 4 compounds were deemed worthy of more intensive investigation. These were BAL itself, the glucoside of BAL (first synthesized by Danielli and co-workers (9), 1-thiosorbitol, and 1-thioxylitol.

REACTIONS BETWEEN MERCAPTANS AND Cd^{++} IN VITRO. The reactions between mercaptans and cationic cadmium which occur *in vitro* would presumably be indicative of the expected interaction of the two agents *in vivo*. A brief study of the chemical reactions between the thiols and Cd^{++} was therefore undertaken.

Reactions with BAL. When solutions of $CdCl_2$ (0.5 M) were added to non-buffered aqueous solutions of BAL (0.05 M), a copious, flocculent, white precipitate formed. The precipitation occurred regardless of the molar proportions of the dissolved reactants. Iodometric titration of filtrates following reaction between Cd^{++} and non-buffered solutions of BAL revealed that 1 mol of BAL was combined for each mol of Cd^{++} added. Electrometric titration of H^+ produced by the reaction of solutions of $CdCl_2$ with molar excesses of BAL gave evidence for the formation of 2 equivalents of H^+ for each mol of $CdCl_2$ added. The endpoint of the electrometric titration was taken at pH 6.0 since at lower hydrion concentrations a second reaction between Cd^{++} and BAL occurs which will be described directly.

The simplest formulation consistent with the observations made may be written as follows:

$$\begin{array}{l} CH_2SH \\ | \\ CHSH \\ | \\ CH_2OH \end{array} + Cd^{++} \rightleftharpoons \begin{array}{l} CH_2S\diagdown \\ |\qquad\quad Cd \\ CHS\diagup \\ | \\ CH_2OH \end{array} + 2H^+$$

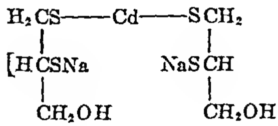
The precipitate formed, hereafter called the Cd-BAL complex, was highly insoluble in water and organic solvents. Although insoluble in alkaline solution, it was dissolved by 0.1 molar solutions of HCl presumably by reversal of the reaction written above.

Of greater importance than the formation of the insoluble Cd-BAL complex, is the reaction which takes place when Cd^{++} is introduced into alkaline solutions of BAL maintained at pH 8.0 or greater by simultaneous addition of strong alkali. So long as the amount of added Cd^{++} did not exceed the molar proportion of 1 Cd:2 BAL, the precipitate which formed in the region of temporary excesses of Cd^{++} was instantly redissolved by agitation of the reaction flask. In a similar fashion alkaline solutions of BAL readily dissolved Cd-BAL which is not soluble in strong alkali alone.

The preparation of a clear solution at pH 8.0 by mixing ½ of a molar equivalent of Cd^{++}

with 0.05 molar BAL required the addition of 1.75 equivalents of NaOH. Further alkalinization caused no precipitation and CdS did not appear when large excesses of Na_2S were added. However, when the pH of the preparation was lowered to 7 or Cd^{++} was added in excess of the molar proportion of 1 Cd:2 BAL, a flocculent, white precipitate immediately formed which presumably was Cd-BAL.

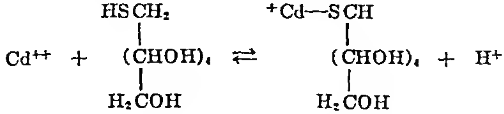
It is apparent in these results that under alkaline conditions 2 mols of BAL react with 1 mol of Cd^{++} to form a soluble complex which is highly undissociated with respect to its components. A simple formulation for the structure of the di-sodium salt of this complex, is as follows:



The complex will hereafter be designated as $Cd(BAL)_2$.

Although the solutions of $Cd(BAL)_2$ described above were prepared from 0.05 molar BAL at a pH of 8 or greater, it should be noted that appreciable amounts of soluble complex can exist in solution at more physiological hydrion concentrations. When a molar equivalent of BAL solution was added to a suspension of Cd-BAL and the mixture neutralized by addition of alkali, clarification was initiated when the pH was elevated to 7.0 and was complete at pH 7.8. This observation would presumably indicate that *in vivo* BAL may be expected to react with Cd^{++} to form a soluble complex and in fact such a reaction has been used below as a reasonable explanation of the changes effected by BAL therapy in the course of cadmium intoxication.

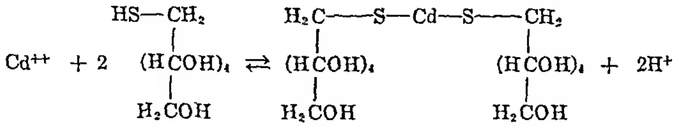
Reactions with thiosorbitol. The thiosorbitol employed in this study was obtained from Dr. B. W. Howk and Dr. W. H. Vinton, of the duPont laboratories. The samples exhibited 96 per cent of theoretical thiol activity. As might be expected, the reactions of thiosorbitol with di-valent cadmium can result in the formation of either the mono-thiosorbitol or the di-thiosorbitol mercaptide of cadmium, depending upon the molar proportions of the reactants. When aqueous solutions of thiosorbitol (0.05 M) and $CdCl_2$ (0.5 M) were reacted in the molar proportion of 1:1, a soluble complex of cadmium formed which was so little dissociated that the presence of S^{--} in the reaction mixture did not result in the precipitation of CdS. The reaction also involved the production of 1 molar equivalent of H^+ as determined by titration to pH 7.0 (glass electrode). These observations may be taken as evidence that all of the Cd^{++} had reacted with thiosorbitol as follows:



The mono-thiosorbitol derivative was readily soluble in an aqueous medium below pH 8.0. When the pH of a 0.05 molar solution of cadmium-mono-thiosorbitol was raised above 8, however, a white precipitate appeared which did not interact with S^{--} to form CdS. Presumably the substance was the base of the complex.

When aqueous solutions of $CdCl_2$ (0.5 molar) and thiosorbitol (0.05 molar) were mixed in the molar proportion of 1:2, the subsequent addition of alkali did not raise the pH to 7 until 2 molar equivalents of H^+ had been neutralized. Inasmuch as thiosorbitol is so weak an acid that the pH of 0.05 molar solutions is raised above 8 by the addition of less than $\frac{1}{20}$

of a molar equivalent of alkali, the above evidence indicates that at pH 7, 2 mols ofthe monothiol combine with each mol of Cd^{++}. This reaction may be depicted as follows:



The complex formed will be designated hereafter as Cd(thiosorbitol)$_2$. Like the Cd-thiosorbitol derivative, it was soluble in water and was not decomposed by addition of S^{--}. Moreover, alkalinization of its squeous solutions caused no precipitation which is further evidence for its non-identity with the mono-derivative.

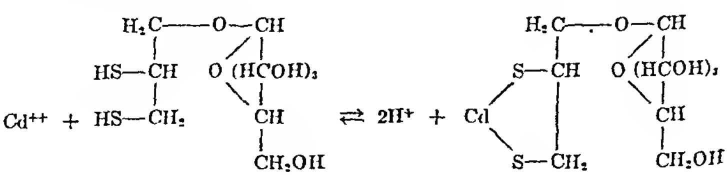
The reactions between Cd^{++} and thioxylitol *in vitro* may be presumed to parallel those with thiosorbitol.

Reactions with BAL glucoside. The samples of BAL glucoside employed in this study were received from Dr. B. W. Howk and Dr. W. H. Vinton of the duPont laboratories. The compound, which was obtained as the barium salt, contained approximately theoretical amounts of Ba and S, but only 60 to 70 per cent of theoretical SH activity. The sodium salt of the glucoside was formed by the addition to solutions of the barium salt of a slight excess of Na_2SO_4 and the pH adjusted to circa 7.4 by the addition of HCl. The $BaSO_4$ was removed by centrifugation and aliquots of the supernatant solution were titrated iodometrically for SH activity. The molar equivalence of the reactions below was calculated in terms of dithiol activity as determined analytically.

Following the addition of 0.5 molar $CdCl_2$ in widely varying molar proportions to neutral solutions of 0.05 molar BAL glucoside precipitation of an insoluble mercaptide did not occur. Therefore, like thiosorbitol, polyhydric BAL glucoside forms heavy metal derivatives with considerable aqueous solubility. When the molar proportionality of the glucoside was equal to or in excess of Cd^{++}, the addition of S^{--} caused no precipitation of CdS. Moreover, no precipitate appeared when solutions containing the heavy metal and BAL glucoside in molar equivalent amounts were alkalinized.

Evidence for the formation of more than one mercaptide as the result of the reactions between Cd and BAL glucoside was obtained from measurements of the acid production resulting from the combination of different molar proportions of the reactants at various pH values. For this purpose, solutions of Ba-free BAL glucoside were first acidified to pH 2.7, which appeared from titrations with strong acid to be the hydrion concentration at which all weak acid radicals were in the undissociated state. $CdCl_2$ was then added to the acidified BAL glucoside in proportions of either ½ or 1 mol:1 mol of dithiol. The reaction mixtures were titrated with NaOH and the amount of acid released by reaction of Cd^{++} with BAL glucoside was compared with the H^+ available from dissociation of the dithiol itself.

Typical results obtained from the above determinations are shown in table 1. It will be noted that only 0.22 molar equivalents of NaOH were required to bring BAL glucoside to pH 7.0 (second column) while 2.00 molar equivalents of alkali were required to bring a $CdCl_2$ and the dithiol to the same pH (third column). The latter result is consistent with the following formulation:



The mercaptide formed is analogous in structure to that previously suggested for insoluble Cd-BAL and is designated hereafter as Cd-BAL glucoside.

It may be further noted in table I that the titratible acidity of the reaction mixture containing only ½ of a molar equivalent of $CdCl_2$ (fourth column) between pH 6.0 and 9.0 was greater than expected as the result of a 1:1 reaction between Cd^{++} and BAL glucoside. In order to account for this observation it is suggested that Cd^{++} can react with 2 molar equivalents of BAL glucoside to form a mercaptide in which the dissociation of the free

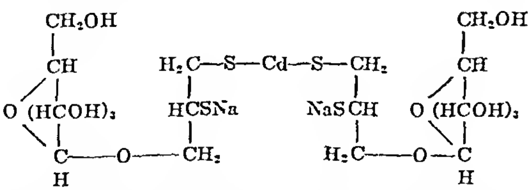
TABLE 1

Titratible acidity of aqueous solutions of BAL glucoside following addition of $CdCl_2$

BAL glucoside, 0.0409 molar, adjusted to pH 2.7 with 1.01 molar HCl (glass electrode, Beckman pH meter); titrated to various pH with 1.01 molar NaOH in the absence and presence of $CdCl_2$; amount of NaOH added expressed as molar equivalents in terms of amount of BAL glucoside present.

pH	BAL GLUCOSIDE, 0.818 mM	BAL GLUCOSIDE, 0.818 mM, + $CdCl_2$, 0.818 mM	BAL GLUCOSIDE, 1.64 mM, + $CdCl_2$, 0.818 mM
	NaOH, molar equiv.	*NaOH, molar equiv.*	*NaOH, molar equiv*
3.0	0.03	1.74	0.96
4.0	0.10	1.83	1.10
5.0	0.15	1.95	1.22
6.0	0.17	1.99	1.31
7.0	0.22	2.00	1.48
8.0	0.50	2.02	1.87
9.0	0.97	2.06	2.01
10.0	1.37	2.15	2.07

thiol groups is increased. By analogy with the structure of $Cd(BAL)_2$ the sodium salt of such a mercaptide may be written as follows:



The product of the reaction between ½ mol of Cd^{++} and 1 mol of BAL glucoside between pH 6.0 and 9.0 is hereafter designated as $Cd(BAL\ glucoside)_2$. It should be recognized that this designation does not imply a final decision as to the structure of the mercaptides found in such a reaction mixture. Solutions containing $Cd(BAL\ glucoside)_2$ give no evidence for precipitation following addition of S^{--} or alkalinization. Acidification presumably converts $Cd(BAL\ glucoside)_2$ to Cd-BAL glucoside similar to the precipitation of Cd-BAL which follows acidification of solutions containing $Cd(BAL)_2$.

THE EFFECT OF MERCAPTANS ON THE COURSE OF SYSTEMIC CADMIUM INTOXICATION. The efficacy of mercaptan therapy on systemic cadmium intoxication was studied in the rabbit. Acute systemic cadmium intoxication in the rabbit runs

a rapid course. Following the intravenous injection of 5.0 mg./Kg. of $CdCl_2$ (1 per cent solution) animals began to show symptoms within 5 to 30 minutes. The rabbits tended to lie quietly in a relaxed position. The respiratory rate was increased and breathing was somewhat dyspneic. An early manifestation of action was increased motor activity of the gastro-intestinal tract which led to the excretion of copious stools which often become watery within the course of an hour. As lethargy progressed, the rabbits assumed unnatural, sprawled posiions, exhibited a marked degree of muscular flaccidity, and eventually became prostrate and appeared to be in a shock-like condition. Death usually ensued in less than 24 hours. Aside from generalized vascular congestion there were no outstanding pathological changes. A rare animal survived for a longer period of time. Usually a rabbit surviving the acute effects recovered completely. In exceptional circumstances, however, late deaths occurred which occasionally were associated with renal or hepatic insufficiency. During the course of studies on antidotal therapy with thiol derivatives, 146 untreated rabbits received 5.0 mg./Kg. of $CdCl_2$ intravenously. Of these 131 died in less than 24 hours; of the remainder, 10 survived (table 5). This dose represents an LD_{93} and was the one employed in all tests of therapy.

When the intravenous dose of $CdCl_2$ was reduced to 3.0 mg./Kg., approximately 59 per cent of animals succumbed. This does was employed in 34 control animals (table 5). Of the 20 which succumbed, 12 died within 24 hours and an additional 4 during the second 24 hours. Of the 4 which survived for longer periods, none showed a significant degree of renal insufficiency as evidenced by the NPN concentration of the blood. Animals which survived indefinitely exhibited no untoward signs during the period of observation (15 days) and in only 2 of these was a temporary elevation in NPN noted.

When the dose of $CdCl_2$ was reduced to 2.5 mg./Kg., a marked reduction in toxicity occurred. Of 12 animals receiving this amount of cadmium, 10 survived (table 5). One died in 18 hours and 1 in 48 hours. There was little evidence of renal insufficiency in this group. The specific data on the effects of a dose of 2.5 mg./Kg. of $CdCl_2$ on renal function will be presented below (table 6) in comparison with those animals receiving $Cd(BAL)_2$.

Effect of prophylactic BAL therapy. When solutions of BAL in propylene glycol were administered intravenously, in the dose of 0.1 mM/Kg., immediately prior to the injection of $CdCl_2$, and therapy continued with two intramuscular injections of 0.1 mM/Kg. each, 1 and 3 hours later respectively, the course of the cadmium-poisoned animals was entirely changed. The acute symptoms described above were usually not observed. Twenty-four hours after the administration of $CdCl_2$ a very high percentage of animals still survived and appeared normal. This was in contrast to the high mortality rate during the first 24 hours in untreated animals. Despite the initial success of therapy, however, delayed death invariably occurred. Of 13 animals receiving 5.0 mg./Kg. of $CdCl_2$, intravenously, and treated as described above, none survived (table 5). However, only two deaths occured within the first 24 hours; the remainder were scattered through the second, third and fourth days. A gross pathological

examination of rabbits dying after BAL therapy revealed, invariably, extensive involvement of the kidneys. These organs were always swollen, soft and flabby and the cortex was pale and pitted by numerous petechial hemorrhages. The microscopic pathology of the kidneys will be the subject of a future communication. Associated with these pathological changes was a functional insufficiency as evidenced by precipitous rises in the concentration of serum NPN.

The absence of the characteristic acute systemic effects of cadmium in the above animals points to the formation of a stable mercaptide with BAL which effectively protects certain sensitive loci from the toxic effects of the metal. However, by virtue of the propensity for the renal excretion of metallo-organic complexes, it would appear that the formation of the $Cd(BAL)_2$ complex *in vivo* directs the toxicity of cadmium toward the kidney.

More striking evidence of the development of renal insufficiency in cadmium-intoxicated animals treated with BAL was obtained when the dose of cadmium was reduced. Under these circumstances a larger number of control animals survived and the influence of treatment in developing the nephrotoxic action was more evident. A total of 15 animals receiving 3.0 mg./Kg. of $CdCl_2$ was treated with 0.3 mM/Kg. of BAL in the manner described above. Of these, 13 succumbed despite treatment (table 5). This represents a higher mortality rate than in the control series of 34 animals receiving this dose of $CdCl_2$ mentioned above. Death characteristically occurred late, only one of the 13 taking place during the first 24 hours. Animals which survived for at least 48 hours invariably showed an elevation in blood NPN (table 2).

Effect of delayed treatment with BAL. A finding which at first appeared paradoxical was that a delay of 30 minutes following the intravenous administration of $CdCl_2$ before BAL therapy was instituted was favorable for survival (table 5). In table 3 are presented the data on 21 rabbits receiving 5.0 mg./Kg. of $CdCl_2$ intravenously, and treated with 0.3 mM/Kg. of BAL in propylene glycol solution in 3 equally divided doses given $\frac{1}{2}$, $1\frac{1}{2}$ and $3\frac{1}{2}$ hours after cadmium, respectively. As can be seen, 10 of 21 animals survived. Inspection of the NPN values of the serum at 96 hours reveals that it was largely the renal status of the animal which determined its ultimate survival. A similar result was obtained in a small series of rabbits when treatment was delayed as long as 60 minutes following intravenous intoxication with 5.0 mg.Kg. of $CdCl_2$ (Table 5). An interpretation of these findings appears in the discussion.

Treatment with thiosorbitol and thioxylitol. The high incidence of renal insufficiency observed in animals treated with BAL could be attributable to the renal reabsorption of the $Cd(BAL)_2$ complex attending its excretion. For this reason mercaptans were sought which might presumably form complexes which would escape renal tubular reabsorption. The therapeutic efficacy of 3 compounds in this category was tested, thiosorbitol, thioxylitol and BAL glucoside.

It has been shown *in vitro* that the monothiols as a group are less effective than the dithiols in reversing the inhibition of enzyme systems by heavy metals (1). Observations *in vivo* on the effectiveness of the monothiols, thiosorbitol and thioxylitol, in preventing death of rabbits acutely poisoned with $CdCl_2$ have

borne out this point of view. Whereas BAL-treated animals seldom died acutely, 3 of 6 rabbits treated prophylactically with thiosorbitol succumbed within the first 24 hours (table 5). Of 28 animals treated 30 minutes after the administration of $CdCl_2$ 20 succumbed, 19 of these within the first 48 hours. Again the majority of deaths occurred acutely. However, it is of significance that of the 11 surviving animals in the above experiments, only 1 showed a temporary elevation of serum NPN to 60 mg. per cent. Thus, in only one instance could death be attributed to renal insufficiency.

TABLE 2

The effect of prophylactic BAL therapy in rabbits receiving 8.0 mg./Kg. of $CdCl_2$, intravenously*

SURVIVAL TIME	NPN (MG./100 CC. SERUM)		
	48 hrs.	72 hrs.	168 hrs.
hours			
19			
96	159	261	
96	198	317	
S†	58.5	55.5	
45			
45			
72	200		
96	66.5	152	
S	122	118	
48			
70			
124		255	
168		206	238
140		110	
168		137	150

* BAL given in 3 equally divided doses, the first, intravenously, 1 minute before $CdCl_2$, and the second and third, intramuscularly, 1 and 3 hours after $CdCl_2$, respectively.

† S = Survival.

Thioxylitol was also relatively ineffective against systemic cadmium poisoning. Of 9 animals treated 30 minutes after the administration of 5.0 mg./Kg. of $CdCl_2$ with 3 equally divided doses of thioxylitol totalling 1.2 mM/Kg., only 2 survived. However, neither of these showed elevated serum NPN.

Effect of BAL glucoside therapy. The effect of either prophylactic or therapeutic treatment with BAL glucoside is shown in table 5. It may be seen that the initiation of therapy immediately prior to the administration of 5.0 mg./Kg. of $CdCl_2$ prevented death in 9 of 10 animals. In order to treat this dose of $CdCl_2$ effectively, it was necessary to inject only a single dose of the dithiol. Moreover,

it is evident that prophylactic therapy with BAL glucoside did not impair renal function in a manner comparable to prophylactic therapy with BAL (table 4). When therapy with BAL glucoside was delayed for 30 minutes the dithiol still proved effective in the dose of 0.3 mM/Kg. Of the 10 animals treated with this dose only one died during the first day. Two others succumbed on the 4th and 5th days, respectively, presumably of renal impairment. Only 1 of 7 survivors exhibited a moderate elevation of serum NPN (table 4). Animals receiving 10.0 mg./Kg. of $CdCl_2$ could not be effectively treated with BAL glucoside, even when the dithiol was administered prophylactically.

TABLE 3

The relationship between survival and extent of renal insufficiency in rabbits receiving BAL therapy following the intravenous administration of 5.0 mg./Kg. of $CdCl_2$

SURVIVAL TIME	NPN AT 96 HOURS (MG./100 CC. SERUM)
hours	
S*	147.0
S	37.6
S	34 7
S	39.3
S	49.5
S	39.2
S	47.0
S	45.0
S	124.0
S	87.5
3½	
24	
24	
120	345.0
144	372.0
144	278.0
144	380.0
168	319.0
168	335.0
192	276.0
288	101.0

* S = Survival.

In view of the effective therapeutic action of BAL glucoside when administered in a total dosage of 0.3 mM/Kg., it was surprising to find that treatment with a total dosage of 0.75 mM/Kg. was less beneficial. Indeed, the larger amounts of the dithiol appeared to shift the acute, toxic action of cadmium toward the central nervous system. This was seen in a group of 10 animals in which therapy consisted of an initial dose of 0.5 mM/Kg. intravenously, followed after 3 hours by 0.25 mM/Kg. intramuscularly. Within 1½ hours after initiation of therapy 3 of the group were seized by violent clonic-tonic convulsions. Six of the other 7 animals exhibited, to varying degrees, gross tremor and hyper-excitability.

Such signs have never been observed to follow intravenous administration of $CdCl_2$, nor were they observed in control rabbits following injections of single doses of as much as 1.0 mM/Kg. of BAL glucoside.

Within 24 hours 6 of the group had succumbed. Although the other 4 appeared somewhat depressed on the day after treatment, they appeared normal within the next two days. One, however, succumbed on the fourth day of the experiment. Serum NPN values of the 3 survivors at 96 hours were normal.

TABLE 4

The effect of prophylactic and delayed therapy with BAL glucoside in rabbits receiving 5.0 mg./Kg. of $CdCl_2$ intravenously

TIME OF INITIATION OF THERAPY	BAL GLUCOSIDE DOSAGE	TIME OF DEATH	SERUM UREA N (MG./100 CC. SERUM)	
			72 hrs.	96 hrs.
min.	*mM/Kg.*	*hrs.*		
−1	0.1*	S‡	8.6	
		S	14.7	
		S	36.1	
		S	9.9	
		S	9.6	
−1	3 × 0.1†	S	66.9	
		S	35.7	
		S	48.8	
		S	47.4	
		144	114.8	
+30	3 × 0.1†	S		
		S		12.1
		24		
		96		
		S		13.8
		S		67.1
		S		55.0
		S		22.2
		S		12.5
		120		122.0

* 0.1 mM/Kg. BAL glucoside, intravenously, immediately prior to the intravenous injection of $CdCl_2$.

† 0.3 mM/Kg. BAL glucoside, in 3 equally divided doses, the 2nd and 3rd of which were given 1 and 3 hours after the first, respectively.

‡ S = Survival.

The anomalous effects of BAL glucoside in high dosage should be considered in the selection of an optimum dose-level of BAL glucoside for the therapy of cadmium intoxication.

The toxicity of the performed mercaptides of BAL, thiosorbitol and BAL glucoside. Further evidence of the mechanism by which mercaptans detoxify heavy metals *in vivo* was afforded by experiments in which preformed mercaptides of cadmium with BAL, thiosorbitol and BAL glucoside were injected intravenously.

When solutions of the soluble $Cd(BAL)_2$ complex were prepared as described above and injected intravenously in rabbits in a dose equivalent to 5.0 mg./Kg. of $CdCl_2$, the subsequent course of intoxication did not differ from that observed following the intravenous administration of $CdCl_2$ (table 7). Despite the low dissociation constant of the complex it nevertheless seemed to afford a readily available source of cadmium. This apparently was the result of a rapid intracellular dissociation of the complex possibly as a result of oxidation. If the dose

TABLE 5

The effect of mercaptan therapy in rabbits following the intravenous administration of $CdCl_2$

DOSAGE*				TIME OF INITIATION OF THERAPY	MORTALITY	TIME OF DEATH		
$CdCl_2$	BAL	Thiosorbitol	BAL glucoside			0–24 hours	24–48 hours	>48 hours
mg./Kg.	*mM/Kg.*	*mM/Kg.*	*mM/Kg.*	*min.*				
2.5					2/12	1	1	
3.0					20/34	12	4	4
5.0					136/146	131	3	2
7.5					14/14	13	1	
3.0	3 × 0.1			−1	13/15	1	3	9
5.0	3 × 0.1			−1	13/13	2	7	4
5.0	3 × 0.1			+30	11/21	3		8
5.0	3 × 0.1			+60	2/6	1		1
5.0		3 × 0.1		−1	2/3	2		
5.0		3 × 1.0		−1	1/3	1		
5.0		3 × 0.4		+30	17/22	15	1	1
5.0		3 × 0.8		+30	3/6	2	1	
10.0			1 × 0.1	−1	5/5	5		
10.0			3 × 0.1	−1	3/5	2		1
5.0			1 × 0.1	−1	0/5			
5.0			3 × 0.1	−1	1/5			1
5.0			3 × 0.1	+30	3/10	1		2

* BAL and thiosorbitol given in 3 equally divided doses except where indicated; the first, intravenously, and the second and third, intramuscularly, respectively 1 and 3 hours later.

BAL glucoside given similarly, with the exception that all injections were intravenous in the animals receiving prophylactic therapy.

BAL administered in propylene glycol (0.5 mM/cc.). Thiosorbitol and BAL glucoside administered in aqueous solution.

of $Cd(BAL)_2$ complex was reduced to 3.0 mg./Kg. the high systemic toxicity still precluded an adequate appraisal of its nephrotoxic action. Of 12 rabbits receiving this dose only 4 survived. The remainder succumbed over a period of time ranging from 18–120 hours (table 7). Without exception, all animals which survived 48 hours showed elevated NPN values. This was in distinction to the group of animals described above which received an equivalent dose of $CdCl_2$ (3.0 mg./Kg.) in which the incidence of renal insufficiency was rare.

When the intravenous dose of $Cd(BAL)_2$ was reduced to 2.5 mg./Kg. the majority of animals survived (table 7). Under these circumstances, the greater toxicity of the complex than of $CdCl_2$ for the kidney became apparent (table 6).

The course of intoxication following the intravenous administration of Cd $(thiosorbitol)_2$ was similar to that which followed the injection of molar equivalents of $CdCl_2$ with the exception that the Cd-mercaptide was slightly less toxic than cadmium itself (table 7). However, in contrast to $Cd(BAL)_2$, of a total of 12 animals surviving the effects of 2.5 or 3.0 mg./Kg., in terms of $CdCl_2$, only one showed a temporary elevation of NPN. Furthermore, only one of 6 animals which survived the dose equivalent to 5.0 mg./Kg. of $CdCl_2$ exhibited an elevated NPN.

TABLE 6

A comparison of the development of renal insufficiency in rabbits following the intravenous administration of molar equivalent doses of $CdCl_2$ and $Cd(BAL)_2$

DOSE OF $CdCl_2$	DOSE OF $Cd(BAL)_2$	TIME OF DEATH	NPN (MG./100 CC. SERUM)	
			48 hours	96 hours
mg./Kg.	*mg. $CdCl_2$/Kg.*	*hours*		
2.5		S*	35.7	30.5
2.5		48		
2.5		S	40.3	36.9
2.5		S	24.1	60.9
2.5		S	24.9	25.8
2.5		S	27.7	31.2
2.5		S	25.8	
2.5		S	44.8	
2.5		18		
2.5		S	34.3	
2.5		S	29.3	
2.5		S	52.8	
	2.5	S	141.0	200.0
	2.5	S	87.6	126.0
	2.5	264	79.0	149.0
	2.5	S	79.0	144.0
	2.5	S	28.1	25.2
	2.5	240	112.0	293.0
	2.5	72	158.0	
	2.5	S	113.0	
	2.5	S	26.7	
	2.5	S	93.4	
	2.5	S	26.5	
	2.5	48	59.4	

* S = Survival.

The intravenous injection of either Cd-BAL glucoside or Cd(BAL glucoside)$_2$ revealed that each of these mercaptides was much less toxic than either $CdCl_2$ or the mercaptides of BAL or thiosorbitol. The mercaptide of higher molecular weight, Cd(BAL glucoside)$_2$ was the less toxic of the two; none of 5 animals died following doses equivalent to 15.0 mg./Kg. of $CdCl_2$. No animal receiving either mercaptide showed signs of renal impairment (table 7).

The treatment of animals receiving Cd(BAL)$_2$ with BAL. Additional information on the behavior of cadmium and mercaptans *in vivo* was obtained when animals receiving a dose of Cd(BAL)$_2$ intravenously were treated 30 minutes later with a course of three injections of BAL given in the usual manner. These

TABLE 7

The toxicity of cadmium mercaptides of BAL, thiosorbitol and BAL glucoside following intravenous administration to rabbits

DOSAGE IN EQUIVALENT AMOUNTS OF $CdCl_2$				MORTALITY	INCIDENCE OF RENAL INSUFFICIENCY*	TIME OF DEATH		
Cd(BAL)$_2$	Cd(thiosorbitol)$_2$	Cd-BAL glucoside	Cd-(BAL glucoside)$_2$			0–24 hours	24–48 hours	>48 hours
mg./Kg.	mg./Kg.	mg./Kg.	mg./Kg.					
2.5				5/12	9/12			5
3.0				8/12	8/8	2	2	4
5.0				12/12		11	1	
7.5				5/5		5		
	2.5			1/6	1/6			1
	3.0			5/12	1/9		3	2
	5.0			16/22	1/6	16		
		5.0		0/10	0/10			
		7 5		2/5			2	
		15.0		2/5		2		
			5.0	0/15	0/10			
			7.5	0/15				
			15.0	0/5				

* In animals surviving 48 hours.

animals did not die acutely as did untreated rabbits given the same dose of Cd(BAL)$_2$, but rather exhibited the same course as rabbits receiving 5.0 mg./Kg. of $CdCl_2$ and treated prophylactically with BAL. Of 16 animals only 4 succumbed within the first 24 hours. All those which survived for 48 hours or more showed marked elevations of serum NPN and eventually died.

Discussion. It is clearly evident that whereas the presence of BAL diminishes the toxicity of cadmium for certain vital tissues it simultaneously enhances the toxicity of the metal for the kidney. A reasonable interpretation of this complication of BAL therapy stems from the observation that suitable concentrations of the dithiol can react with Cd^{++} in the physiological pH range to form a soluble, stable mercaptide, Cd(BAL)$_2$. Presumably the formation of Cd(BAL)$_2$ can

effectively direct significant amounts of the metal away from those tissues upon which cadmium acts to cause early collapse and acute death. However, by virture of its solubility and the propensity for metallo-organic complexes to be excreted by the kidney appreciable quantities of the mercaptide are made available for glomerular filtration and tubular concentration. A significant degree of tubular absorption would result in concentration of the complex in the tubular epithelium and consequent intoxication of these cells due to the intracellular release of Cd^{++}.

The interpretation that $Cd(BAL)_2$ can undergo intracellular decomposition into its components would seemingly belie its supposed stability. This difficulty may be resolved if the protective mechanisms of BAL therapy are visualized dynamically. $Cd(BAL)_2$, while stable *per se*, must be considered as existing in equilibrium with small amounts of free Cd^{++} and BAL. As a consequence of this equilibrium and the probable susceptibility of BAL to intracellular oxidation—a fact attested to by the observation of Barron and coworkers (10) that BAL is readily oxidized *in vitro* by the cytochrome C-cytochrome oxidase system—there is present *in vivo* a mechanism for the continuous destruction of $Cd(BAL)_2$. So long as excess BAL is present intracellularly and in the circulation for a suitable period of time, the concentration of free cationic cadmium will remain low and *cadmium in complex form will eventually find its way to the kidney. A significant* reduction of tissue cadmium is thereby accomplished. The necessity of maintaining an adequate concentration of BAL over an extended period of time in order to remove cadmium from sensitive extrarenal tissues is apparent in the observation that the administration of $Cd(BAL)_2$, equivalent in dosage to 5.0 mg./Kg. of $CdCl_2$, results in rapid collapse and death typical of acute cadmium intoxication. However, most animals receiving such a dose of $Cd(BAL)_2$ and subsequently treated with BAL escape acute intoxication only to succumb eventually as a result of renal insufficiency.

It follows from the above discussion that tubular reabsorption of $Cd(BAL)_2$ will cause the accumulation of intolerable amounts of cadmium within tubular cells. Although in tubular cells the decomposition of $Cd(BAL)_2$ may result from intracellular oxidation, a second mechanism of intoxication is conceivable.

In their investigations with *in vitro* preparations of succinoxidase, Barron and Kalnitsky (5) found that the reversal by dithiols of heavy metal inhibition became difficult at concentrations of inhibitors producing more than 90 per cent inhibition. For example, bismuth at a concentration of 1.2×10^{-5} molar inhibited enzyme activity by 87 per cent. The addition of a dithiol in the molar proportion to Bi of 1.6:1 effected a 75 per cent reversal. However, when the Bi concentration was increased to 2.5×10^{-5} molar, the resulting inhibition was 95 per cent and the reversal by a 1.6:1 proportion of dithiol was only 14 per cent. Thus, a two-fold increase in inhibitor concentration reduced markedly the effectiveness of the mercaptan. A similar mechanism may lead to the poisoning of tubular epithelium in which the concentrating action of the normal processes of filtration and reabsorption may raise intracellular cadmium levels to such a degree that BAL fails to render protective action.

The above interpretation of the mechanisms involved in BAL therapy of

systemic cadmium poisoning does not imply that the dithiol mobilizes all of the cadmium which has gained access to the tissues of the intoxicated animal. Such a conception would fail to account for the differing results obtained with prophylactic and delayed treatment. The fact that delaying the initiation of therapy reduces the extent of renal damage indicates that in the absence of BAL significant amounts of cadmium are rapidly and irreversibly fixed in tissues whose function remains relatively unimpaired by the presence of the metal. Bunting and coworkers (11) have noted that much of the cadmium which gains access to the circulation following the exposure of dogs to aerosols of $CdCl_2$ can be detected in healthy tissues throughout the body as late as 15 weeks after gassing.

It would follow from the above discussion that a thiol, capable of forming a complex that would remain extracellular and not be reabsorbed by the renal tubule, would prove to be an effective therapeutic agent. In view of the fact that thiosorbitol is a thiol derivative of a hexitol which is not reabsorbed by the renal tubule (12) it might be expected that complexes of this thiol and heavy metals likewise might fail to undergo tubular reabsorption. The data presented above are in agreement with this concept inasmuch as treatment with this compound has not resulted in the degree of renal insufficiency characterizing treatment with BAL. Presumably, however, $Cd(thiosorbitol)_2$ undergoes cellular decomposition inasmuch as the symptoms of acute cadmium intoxication follow the administration of the preformed complex.

Despite the absence of enhanced renal toxicity, thiosorbitol proved even less effective than BAL in the delayed treatment of cadmium poisoning. It was not entirely unexpected that a monothiol, thiosorbitol, should behave in this manner. Indeed, it was the inadequacy of monothiols in reversing arsenic linkage with proteins that led British workers to the trial of dithiols as possible antidotes for lewisite poisoning (1). When considered in conjunction with previous investigations on the treatment of arsenical poisoning, the present results indicate that monothiols are likely to prove relatively ineffective in the treatment of poisoning caused by other heavy metals.

The superiority of BAL glucoside as a therapeutic agent can in part be attributed to the facility with which its mercaptides are excreted by the kidney. If this were the only mechanism involved in the greater efficacy of BAL glucoside, however, it would be difficult to account for the greatly decreased toxicity of its preformed mercaptides. In this regard it is reasonable to propose that the mercaptides of BAL glucoside are relatively stable *in vivo* by virtue of cellular impermeability to the dithiol. In order to account for the relatively low toxicity of the dithiol, Danielli and coworkers have already proposed that BAL glucoside is distributed extracellularly (9).

The concept that BAL glucoside is mainly limited to the extracellular fluid compartment is not contradicted by the observations that the agent is therapeutically effective. Theoretically, it seems justifiable to consider that the intracellular combination of heavy metals with cellular proteins would be to some extent dissociable. Thus, small quantities of the free metallic ion would be in equilibrium with the metallo-protein compound and the equilibrium could be expected to extend beyond cellular borders. It follows then that an extra-

cellular compound, like BAL glucoside, of high affinity for the metal could disturb the equilibrium in favor of the effective removal of the metal from its intracellular combinations. Danielli and coworkers (9) have similarly accounted for the efficiacy of BAL glucoside. In addition they suggest that naturally occurring sulfhydryl substances like glutathion form mercaptides intracellularly and thus serve as intermediates in the diffusion of metals across cell membranes. Until further evidence appears in support of the latter hypothesis it seems reasonable to assume the diffusion of the metals as free ions.

It is of interest to compare the antidotal actions of mercaptans with respect to their efficacy in the treatment of systemic mercury and cadmium poisoning. Mercury, a highly nephrotoxic metal is innocuous in the face of BAL therapy (7) whereas cadmium exhibits only a negligible toxic action on the kidney unless in mercaptide form. In the case of each metal relatively stable mercaptides are formed with BAL and related compounds. The answer may lie in the relative dissociabilities of the mercaptides of cadmium and mercury formed with BAL on the one hand, and the sulfhydryl groups of essential enzymes on the other. Also to be considered is the possibility that Cd^{++} may catalyze the oxidation of the dithiols.

SUMMARY AND CONCLUSIONS

1. Of a total of 44 mercaptans which were screened for their therapeutic efficacy in the treatment of systemic cadmium poisoning in the rabbit only 4 compounds, the dithiols BAL and BAL glucoside, and the monothiols 1-thiosorbitol and 1-thioxylitol, proved sufficiently promising to warrant more intensive investigation.

2. The prophylactic administration of BAL to rabbits which received intravenous lethal doses of $CdCl_2$ resulted in the amelioration of the signs of acute intoxication. However, the therapy was without benefit due to the development of fatal renal insufficiency. When BAL therapy was delayed 30 or 60 minutes following injection of cadmium, a significant reduction in mortality ensued. Here again acute cadmium intoxication was allayed and animals which succumbed exhibited extensive renal insufficiency.

3. The administration of thiosorbitol or thioxylitol, either prophylactically or after a delay of 30 minutes following $CdCl_2$ benefitted only a limited number of animals. Most treated animals succumbed acutely in a manner typical of acute cadmium poisoning. However, survivors did not show an undue incidence of renal damage.

4. Therapy with BAL glucoside, whether initiated immediately prior to or after a delay of 30 minutes following the administration of $CdCl_2$, successfully prevented poisoning in most animals. Furthermore, the incidence and degree of renal damage in surviving animals was significantly less than had previously been observed following therapy with BAL.

5. In order to elucidate the interactions which presumably occur between the mercaptans and cationic cadmium *in vivo* the reactions between BAL, thiosorbitol, and BAL glucoside and cadmium were studied *in vitro*. Tentative formulae have

been assigned to the mercaptides formed *in vitro* and the toxicities of several of the complexes were determined by intravenous administration.

a. $Cd(BAL)_2$ proved to be as acutely toxic as Cd^{++} on a molar basis and in addition exhibited a marked nephrotoxic action.

b. $Cd(Thiosorbitol)_2$ was only slightly less toxic than $CdCl_2$ and resembled the latter in that animals which were fatally poisoned succumbed acutely while survivors usually failed to develop signs of renal insufficiency.

c. The mercaptides of BAL glucoside, Cd-BAL glucoside and $Cd(BAL\ glucoside)_2$ were found to be no more than $\frac{1}{2}$ or $\frac{1}{5}$ as toxic as $CdCl_2$, respectively. Furthermore, relatively high doses of the mercaptides did not evidence nephrotoxic action.

6. In the light of the above results it was concluded that

a. BAL interacts with Cd^{++} *in vivo* to form a mercaptide of low dissociation which is susceptible to intracellular oxidation but which, in the presence of excess BAL, directs the metal toward the kidney for excretion and thereby prevents poisoning of sensitive extrarenal loci; the mercaptide, however, by glomerular filtration and tubular reabsorption is concentrated in the epithelium of the renal tubule where intracellular oxidation results in the release of toxic amounts of Cd^{++};

b. BAL glucoside forms *in vivo* mercaptides of low dissociation which remain extracellular, are directed toward the kidney for excretion, and are not reabsorbed in the tubule to the degree evidenced by $Cd(BAL)_2$;

c. The therapeutic inefficacy of thiosorbitol and thioxylitol in the treatment of systemic cadmium poisoning may be attributed to the decreased ability of monothiols in general to reverse heavy metal linkage with sensitive proteins.

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THE PATHOLOGY AND THERAPY WITH 2,3-DIMERCAPTOPROPANOL (BAL) OF EXPERIMENTAL Cd POISONING

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Cadmium poisoning, although sporadic following food contamination (1) (7), is most common and severe after exposure, mainly in industry, to the substance as a dust or fume (1) (2) (18) (20). Further, the disease produced by para-pulmonic administration is quite different from that following inhalation. Yet, until a careful review in 1932 (18) called attention to the situation, only a single experimental study (14) dealt with inhaled cadmium; and until now the reported work has been meager, although the general text books list Cd, Pb, Hg, As, Cr and Sb as the important metallic dust hazards (11) (18). Prodan's experiments (19), as well as various clinical reports (1) (2) (4) (7) (18), emphasize symptoms, referable mainly to the lungs, partly to the gastro-intestinal tract, and pathological changes, in the lungs supplemented with limited damage to the liver and kidneys. No analysis of the basic chemical pathology was attempted nor was the possibility of therapy explored.

The present report deals primarily with the pathology and treatment, including specific therapy with BAL,[2] of experimental poisoning by inhaled cadmium and is part of an extensive investigation.[3] Details of exposure (21) and analytic methods (23), of body distribution (17), of chemical action (16), and of functional disturbances (22) will be reported separately; here, a minimum of such information is included for orientation.

METHODS. Cadmium was administered to unanaesthetized mice and dogs by inhalation, or occasionally by intravenous, subcutaneous or intraperitoneal injection. $CdCl_2$ was used mainly, but oxide or sulphide dusts were sometimes inhaled and other compounds injected. For exposure by inhalation, mice were kept for twenty to forty minutes and dogs for forty to seventy minutes in a 900 liter dynamic chamber into which a Cd solution was atomized or dust blown. Cd concentration in the chamber air was followed, in aspirated samples, by colorimetric determination as CdS, particle counts, and Geiger counter measurements with Cd^{115}, which gave results in satisfactory agreement. An average Cd concentration in the range 0.04 to 0.14 mg./l. was used for mice; 0.06 to 0.21 for dogs. In therapy experiments, animals to be treated and their untreated controls were exposed simultaneously.

For histologic study, tissues were fixed in 10 per cent neutral formaldehyde and routinely stained with hemotoxylin and eosin. Cd distribution in the body was followed by means

[1] This work was done as part of a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The University of Chicago. The experimental work reported here was done in 1943–44.

[2] BAL, British anti-lewisite-2,3-dimercaptopropanol (15).

[3] This team was one of four studying mechanism of cadmium poisoning in this country in 1943–44. The work led by Dr. E. S. G. Barron at The University of Chicago, Maj. A. Z. Gilman at Edgewood Arsenal, and Dr. H. E. Harrison at Yale can be given only passing mention here (see 25).

of the radioactive isotope Cd^{115} (2.5 day half life), made available, along with counting equipment, through Dr. K. S. Cole. The many thiols tested were supplied by the National Defence Research Committee.

RESULTS. *Physiological pathology in untreated animals.* Following inhalation of $CdCl_2$ mists, dogs die early, within 48 hours or so, of pulmonary edema, or late, in a week to a month, with pneumonia and bronchopneumonia or fibrous pneumonitis, anorexia, bloody diarrhoea and wasting.

The early deaths with pulmonary edema occur, on the average, about twelve hours after exposure. As blood fluid is lost to the lungs there is hemoconcentration, and an accompanying rise (of over 40 per cent) in plasma protein concentration. The hematocrit reaches a maximum about 10 hours after exposure, rising 50 per cent on the average but more than this in the earlier fatalities. Both changes are minimal in surviving animals and provide a basis for prognosis as to early death. Blood NPN is increased only in proportion to the hemoconcentration. Arterial blood oxygen saturation falls to 50 per cent, on the average, and venous to 13 per cent at half an hour before death from edema. Respiratory rate increases markedly and temperature rises irregularly. An early bradycardia passes over into tachycardia; arterial and venous blood pressures both fall, but rarely severely. The scleral circulation shows dilated arterial tips, open arterio-venous anastomoses, and an irregular, sluggish blood-flow. Erythrocytes are moderately clumped intravascularly, but fragility is not abnormal.[4] After death, fluid pours from the nose and mouth. Its average protein content, 5.3 per cent is about half the terminal plasma concentration. Lung weight, in the dog, averages 3.6 per cent of the body weight (normal 1.1 per cent), but ranges from 1.5 to 5.5 per cent and correlates poorly with severity of exposure. In mice, lung weight at death is fairly uniform at 2 to 2.5 per cent of body weight (normal 0.6 per cent); it rises progressively after exposure as edema develops.

Prodan (19) has made similar observations on cats exposed to cadmium sulphide or oxide dusts. After exposure to high concentrations the more severe symptoms are those leading to death from pulmonary edema; in animals dying late after less severe exposures, the dominant lung effects are those of pneumonia with some scar tissue formation.

Structural pathology in untreated animals. Mice. The temporal sequence of pathological change after $CdCl_2$ inhalation was studied in greatest detail in mice, which were sacrificed in groups at intervals up to 100 hours after exposure. The severity of each type of lesion was assigned an arbitrary value and the average for each type was plotted against time (fig. 1). Although the quantitative limitations of such curves are obvious, they will be found of value in following the discussion below.

The earliest recognizable lung change is perivascular, peribronchiolar edema, seen as soon as three hours after exposure and becoming progressively more marked for 36 hours or so. The bronchioles and bronchi appear to be encased in a thick sheath which is, in reality, perivascular connective tissue distended

[4] For *in vivo* observation of the capillary circulation we are indebted to Dr. E. H. Bloch, now at Michael Reese Hospital, Chicago.

with protein-containing fluid. Between 36 and 48 hours this edema may begin to subside, but inflammatory cells are then numerous. Alveolar edema, on the other hand, is seen first about 24 hours after exposure, commonly around the alveolar ducts and atria, and during the next 24 hours it becomes diffusely distributed throughout the lungs.

Pulmonary infiltration by inflammatory cells first appears after the perivascular and before the alveolar edema. At 14 to 16 hours after exposure, polymorphonuclear leucocytes increase throughout the lung, first in alveolar walls and perivascular connective tissue, but later, marked in 36–48 hours, becoming most

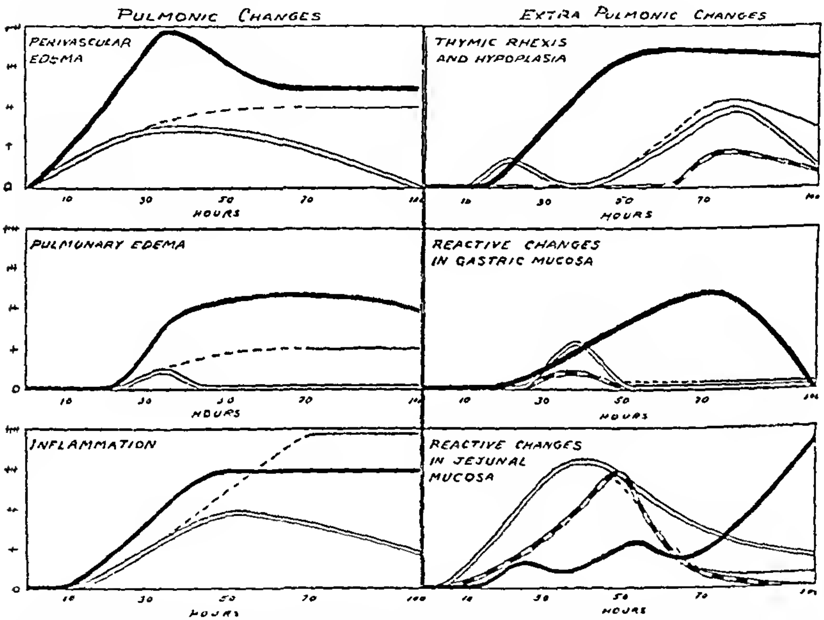


FIG. 1. Heavy solid line—Cd inhalation, no treatment. Double line—Cd inhalation BAL treated survivors. Thin solid line—Cd inhalation, BAL treated fatalities. Dotted line—interpolations on above. Dashed line—no Cd inhalation, BAL treatment controls.

numerous in the bronchiolar walls, epithelium and lumina, and in the alveoli adjacent to bronchioles and alveolar ducts. As time goes on, fibroplasia and organization begin in these areas. Actual lobar consolidation, suppurative bronchiolitis and abscess formation may occur due to secondary infection of the damaged tissue. Macrophages and fibroblasts are slow to appear. No real change in the character of exudate occurs up to about four days after exposure.

The fact that edema is first seen in the peribronchiolar, perivascular connective tissue, and not until somewhat later in the alveoli, does not necessarily mean that it actually starts to form first in the peribronchiolar tissue. Pulmonary lym-

phatics extend along peribronchiolar arterioles only to the alveolar ducts (12), and edema fluid formed by leakage from alveolar vessels would be drained away by these lymphatics. Progressively more heavily loaded, and almost surely also damaged by the toxic agent, these lymph channels would soon reach their maximum functional capacity and then dilate and leak, thus producing the perivascular, peribronchiolar edema. Perhaps only then, when they could no longer successfully drain the forming edema fluid, would visible exudate begin to accumulate in alveolar structures. Alveolar vascular leakage may, thus, first manifest itself as edema of peribronchiolar connective tissue.

Thymic and occasionally splenic lymphocytes begin to fragment at 16 to 24 hours after exposure, the lymphorrhexis being essentially complete by 48 hours. Thymic reticulo-endothelial cells undergo hypertrophy and hyperplasia and phagocytize the cellular debris. The thymus almost disappears and finally consists only of a few normal lymphocytes, scattered in reticulo-endothelial cells and connective tissue. Intestinal and gastric epithelial cells in the depths of the glands undergo non-specific reactive changes similar to those which occur after X-irradiation or poisoning with mustard gas or the nitrogen mustards. These consist of nuclear vesiculation and enlargement, condensation of chromatin into large nucleoli, and swelling and clearing of the cytoplasm. There is also a significant increase in the number of mitotic figures. Though starting early, these changes are not prominent until about 70 hours after exposure. The estimate (17) that over 95 per cent of inhaled cadmium is excreted (in untreated animals) into the intestinal lumen by the gut wall and the liver is significant in this connection.

No consistent changes were found in the heart, liver, spleen, bone marrow, adrenals, brain or kidney.

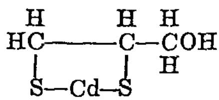
Dogs. Although fewer dogs were examined, the temporal sequence of events after $CdCl_2$ inhalation appears to be much like that in mice. The lung damage seen was more severe than in mice; but this may have been due to a greater retained dose (dogs retain a higher percentage of inhaled particles (21)) and to preexistent lung infection. Polymorphonuclear leucocytes and occasional macrophages appear everywhere and become numerous in the tracheal and submucosal veins. Some cells infiltrate the epithelium, more the peribronchial connective tissue and bronchial musculature. Infiltration is maximal in the bronchioles, and may lead to occlusion of alveolar ostia. The bronchiolar epithelium frequently is necrotic or entirely sloughed off. Alveoli are usually completely filled with protein-rich edema fluid. The intestinal tract shows reactive changes similar to those described for the mouse, and the liver Kupffer cells are more prominent than normally. The heart, kidney and spleen are normal in appearance.

Intraperitoneal administration of 1 to 10 mg. of Cd per kg. results in similar reactive changes in the intestine and swelling of the Kupffer cells, whereas intravenous administration of the same amount produces no typical structural changes.

As will be seen, the structural pathology, like the body distribution of Cd, is markedly changed by therapy with BAL.

THERAPY. *Rationale.* The action of cadmium ions on animal tissues was assumed to be analogous to that of other heavy metal ions, which combine with tissue protein carboxyl or sulfhydryl groups (15) to form insoluble metal proteinates or mercaptides and so impair enzyme and other cellular function. Dithiols had been demonstrated (15) to be effective in preventing or reversing such combination in the case of As and were a first choice in seeking a specific therapy for Cd. BAL proved effective at once (July, 1943), but with a lower margin of safety than for As. Further extensive study, therefore, was made of dosage, of other thiols (monothiols have some value, unlike the case for As), of non-thiols and of chemical mechanisms. Cd, for example, inhibits some—SH enzymes but spares others (3) (16); and its *in vitro* action is reversed to varying degrees by thiols. Actually, BAL proved to be the most satisfactory agent for treating Cd poisoning, as it had earlier for As (15) and did later for Zn (8) and Hg (9a).

Presumably cadmium is removed from vital tissue components by transformation into



(Cd-BAL), and this is excreted mainly through the kidneys (17). Cd-BAL was prepared (16) and, as expected on the basis of stability and insolubility, proved far less toxic than $CdCl_2$. The latter, given intraperitoneally in a dose of 10 mg. Cd per kg., killed 90 per cent of the mice within 24 hours; whereas 33 mg. Cd per kg., given as Cd-BAL, killed none and 55 mg. per kg. killed only 20 per cent. A soluble $Cd(BAL)_2$, however, was found to be highly toxic to the kidney (9b).

Toxic effects of BAL alone. Mice tolerate single doses of 40 mg. of BAL per kg. given intraperitoneally or intramuscularly (table 1). There are no signs of acute poisoning at this dose. Delayed deaths were seen in some early experiments, probably due to use of infected mice (p-typhoid). Four 40 mg. doses (table 2), given at three hour intervals, kill 5 to 10 per cent of the animals, the deaths occurring within a few hours. Dogs (table 3) tolerate single doses up to 75 mg. per kg., given intraperitoneally or intravenously. When repeated four times at three hour intervals, 30 mg. per kg. may cause some late deaths (1–3 weeks); 37 mg. per kg. so repeated may be fatal in a day.

BAL acts somewhat more rapidly following intravenous or intraperitoneal than intramuscular injection. On percutaneous inunction in dogs or mice (solution or ointment), larger amounts of BAL are necessary to produce toxic effects; a dose of 100 mg. per kg. given in a jelly base is well tolerated, and even six such doses at two hour intervals do not lead to acute deaths. Given by stomach tube, 120 mg. BAL per kg. is well tolerated by mice, but even 40 mg. per kg. repeated 4 times kills about 40 per cent of the animals in 5 days. Two dogs died following administration by stomach tube of two doses of 62 or 124 mg. per kg. at a three-

hour interval. Mice apparently can tolerate 30 to 40 minute exposures to atomized BAL in concentrations of 0.3 to 0.6 mg. per liter. More severe exposures cause some deaths. Atomization with air does not destroy sulfhydryl groups.

Details concerning the use of BAL in arsenical poisoning in humans have been published (24) (25). It is given intramuscularly, as a 10 per cent solution in oil, 0.025 cc. (2.5 mg.) per kg. repeated 4 times at 4 hour intervals during the first day

TABLE 1

Toxicity of BAL for mice—single dose

NO. OF MICE	DOSE	ROUTE	PER CENT MORTALITY	
			2 days	5 days
	mg./kg.*			
3	40	Intravenous	0	66
3	80	Intravenous	0	33
3	120	Intravenous	66	100
39	12	Intraperitoneal	16	33
44	25	Intraperitoneal	15	68
60	50	Intraperitoneal	25	35
37	75	Intraperitoneal	50	72
17	100	Intraperitoneal	77	77
31	75	Intramuscular	32	
20	100	Intramuscular	75	75
2	40	Oral	0	0
2	80	Oral	0	0
2	120	Oral	0	0
10	25	Percutaneous	20	40
10	50	Percutaneous	0	0
10	100	Percutaneous	0	30
5	8 (0.8 × 10)†	Inhaled	0	0
10	13 (0.3 × 44)	Inhaled	0	0
5	21 (0.6 × 35)	Inhaled	0	0
6	27 (0.45 × 60)	Inhaled	80	80
6	40 (0.50 × 80)	Inhaled	17	17
6	80 (0.40 × 200)	Inhaled	0	0

* BAL given in 0.2 cc. saline, except oral 0 4 cc.

† Notation indicates—concentration × time (mg./l. × mins.).

and once daily for the following six days. Minor toxic reactions, such as nausea, generalized aches and pains and a burning sensation in the mouth and eyes, occur in about 1 per cent of the cases, but disappear within 1 to 4 hours. Comparable but more severe symptoms are shown by dogs dying a few hours after BAL administration. There is an initial period of decreased irritability followed by vomiting, lachrymation, dyspnoea, convulsive seizures, ataxia, hyperpnoea, and

salivation alternating with periods of comparative normality. Death usually occurs as a result of respiratory arrest in a convulsive seizure. Essentially similar findings have been reported for cats (5). Dogs given BAL orally may also have rather violent diarrhoea. A few dogs died several weeks after BAL injection, but not certainly because of BAL, with severe emaciation, depression, and terminal convulsions. Symptoms are more difficult to evaluate in mice, but toxic doses lead to depression of activity followed, in animals given high doses, by increased irritability, muscle twitching, gross convulsive seizures and death.

Jejunal pathology produced by BAL alone is much like that induced by Cd, but there is far less reactive change in the gastric mucosa. The jejunal reactions are

TABLE 2

Toxicity of BAL for mice—repeated doses

NO. OF MICE	AMOUNT GIVEN				ROUTE	PER CENT MORTALITY	
	Each dose	No. of doses	Dose interval	Days given		2 days	5 days
	mg./kg.		*hrs.*				
30	40	3	3	1	Intraperitoneal	17	17
12	40	4	3	1	Intraperitoneal	0	8
24	20	6	2	1	Intramuscular	7	17
8	20	6, 2	2, 12	1, 2nd, 4th	Intramuscular	0	12
25	20	6, 4	2, 3	1, 2nd, 5th	Intramuscular	20	28
5	25	8	2	1	Intramuscular	0	10
53	40	4	3	1	Intramuscular	6	4
24	40	3, 2, 1	3, 6	1, 2nd, 3rd, 4th	Intramuscular	4	20
5	40, 20	1, 4	2	1	Intramuscular	20	20
5	40, 20	1, 4	2	1 and 3rd	Intramuscular	20	40
5	40, 20	1, 4	2	1, 3rd and 5th	Intramuscular	20	100
5	40, 20	1, 4	2	1 and 5th	Intramuscular	0	20
5	40, 20	1, 4	2	1 and 7th	Intramuscular	0	20
9	40	4	3	1	Oral	10	45

marked for only a short time, at about 48 hours after administration (compared with 70 hours for Cd), after which they subside.

Effect of BAL on mortality from cadmium poisoning. Almost all (94 per cent) mice given 10 mg. Cd per kg. intraperitoneally die within 24 hours if untreated (table 4). In early experiments, repeated doses of BAL given intramuscularly prevented 50 per cent of such deaths at 24 hours, and 30 per cent of the mice were alive at 5 days. In the final experiments, when an optimal course of BAL treatment had been developed, 7 per cent of the untreated animals and 93 per cent of the treated ones were alive at 14 days after cadmium inhalation (table 5).

In the search for a satisfactory course of BAL therapy, 2500 mice were exposed to $CdCl_2$ for an average of 30 minutes (range 20 to 40) at an average Cd concentration of 0.15 mg. per liter (range 0.02 to 0.77). Subsequently, 1186 of these

animals were followed as controls on mortality, 1314 were given varying courses of treatment with BAL (table 5). The overall results, with all BAL experiments,

TABLE 3

Toxicity of BAL for dogs

NO. OF DOGS	AMOUNT GIVEN					SURVIVAL
	Each dose	No. of doses	Dose interval	Route	Vehicle	
	mg./kg.		*hrs.*			*hrs.*
6	25–80	1		Intravenous	Saline	Lived
1	100	1		Intravenous	Saline	2
1	120	1		Intravenous	Saline	0.5
2	20	4	3	Intravenous	Saline	Lived
2	25	4	3	Intravenous	Saline	Lived
4	25–50	1		Intraperitoneal	Saline	Lived
1	120	1		Intramuscular	Saline	4
1	13	3, 5	1, 2	Intramuscular	Saline	Three weeks
1	13	14	2	Intramuscular	Saline	Lived
5	30	4	3	Intramuscular	Saline	One to three weeks
3	40	4	3	Intramuscular	Saline	9–22
1	50	2	1½	Intramuscular	Saline	Lived
1	60	2	3	Oral	Saline	7
1	120	2	3	Oral	Saline	15
1	120	1		Intramuscular	Saline	3
3	150	1		Intramuscular	Saline	3
1	120	1		Intramuscular	Beeswax—Sesame oil	Lived
1	200	1		Intramuscular	Beeswax—Sesame oil	12

TABLE 4

BAL treatment of extrapulmonic cadmium poisoning in mice*

(Non-optimal course of BAL treatment)

NO. OF MICE	BAL THERAPY				PER CENT MORTALITY AT DAYS INDICATED		
	Route	Dose	No. of doses	Dose interval	1	2	5
		mg./kg.		*hrs.*			
70		0			94	94	95
60	Intramuscular	40	4	3	45	56	66
30	Intraperitoneal	40	1	Immed.	70	83	83

* All animals given 10 mg. of Cd per kg. intraperitoneally as $CdCl_2$ in saline.

showed an extra survival with treatment; of 32 per cent at two days and 25 per cent at two weeks. Statistical analysis, by Dr. Sewall Wright (table 6), con-

firmed the impression that 120 (−160) mgm. BAL per kg., given in 3 (−4) to 6 (−8) divided doses over a 12 (−16) hour period, was about optimal. Smaller total amounts of thiol were less effective, distinctly larger amounts, deleterious. (In one series, survival was somewhat better with 8 doses than with the standard 6.) BAL, 10 per cent in 0.9 per cent saline, was injected intramuscularly in all these tests. Such treatment prevented about three-fourths of the deaths expected by four days, half of those expected at two weeks. Actually the therapeutic results are considerably better than these data suggest, for many of the experiments were complicated by a tardily recognized disease in the mice obtained from several of the suppliers. Due to a p-typhoid-like infection, the death rate in stock animals, kept under like conditions but given no Cd or BAL,

TABLE 5

BAL treatment of mice poisoned by inhalation of cadmium chloride mist*

NO. OF MICE	BAL THERAPY	PER CENT MORTALITY AT DAYS INDICATED			
		2	4	9	14
Stock strain 60		0	7	20	45
Cadmium					
1186	None	55	85	93	95
1314	Various†	23	49	64	70
220	None	44	75	80	84
189	20 mg./kg. intramuscularly in saline every 2 hours for 6 doses	8	18	36	44
15	20 mg./kg. intramuscularly in saline every 2 hours for 8 doses	0	7	20	41
15	None	53	93	93	93
15	20 mg./kg. intramuscularly in saline every 2 hours for 6 doses	7	7	7	7

* Aver. conc'n. Cd. = 0.15 mg./l.; aver. exposure = 30 minutes.

† Exploratory experiments to determine optimal course of treatment.

was at times as high as 3.1 per cent per day. As shown in figure 2 and table 5, deaths due to Cd are essentially complete at four days and, at this time, over 80 per cent of the expected Cd deaths were prevented by BAL treatment. Later deaths in the Cd-poisoned, BAL-treated group were actually at a lower rate than in the stock animals. Further, the lung pathology in all these later casualties was that of pneumonic infection and not related to the cadmium picture. Finally, a series of experiments with presumably healthy mice gave an end result of 7 per cent untreated and 93 per cent treated mice surviving (table 5).

Treatment must be instituted soon after exposure, 1 to 3 hours, to be effective (table 6). BAL, begun 3 to 6 hours after exposure in a few experiments, had but a dubious effect in mice and, begun between 6 and 12 hours, seemed actually to

hasten death. A single dose (20 to 40 mg. per kg.) increases survival time and number, but is definitely less effective than a course of injections. BAL given orally (stomach tube) or percutaneously (abdominal skin) is also less effective for mice than when injected intramuscularly. Atomized BAL is ineffectual in both dogs and mice. BAL given as a prophylactic (one injection of 40 mg. per kg.) before exposing mice to $CdCl_2$ mist is distinctly detrimental (table 6). It leads

TABLE 6

Analysis of BAL treatment results

TREATMENT	BAL	NO. OF DOSES	NO. OF EXP'TS.	PER CENT ALIVE AT FOUR DAYS	SURVIVAL AT FOUR DAYS; MEAN ± S.E. (TRANSFORMED SCALE)	CONCLUSION
	mg./kg.					
Normal control	0	0	6	93	79.2 ± 5.3	
Cadmium control	0	0	25	15	20.4 ± 2.4	
BAL control	20–40	varied	15	80	67.4 ± 6.0	BAL deleterious by itself.
BAL therapy	40	2 or 3	5	77	64.4 ± 6.5	2 or 3 doses of 40 mg./kg. or about 6 doses of 20 mg./kg. is effective.
	20	6	12	76	63.2 ± 4.2	
	40	1	6	72	58.5 ± 5.9	
	20	3 or 4				
	20	7 to 10	4	67	56.3 ± 7.2	more than 6 doses of 20 mg./kg. is probably deleterious.
	40	4	10	57	48.4 ± 4.6	
	40	2 or more	10	55	48.2 ± 4.6	
	20	several	10			
	20	2 to 4	2	53	46.5 ± 10.2	2–4 doses of 20 mg./kg. is not enough.
	6–12	6	3	46	43.0 ± 8.4	6 doses of 6–12 mg./kg. is relatively ineffective.
BAL (delayed)	20–40	3 or 4	9	22	25.3 ± 4.6	BAL ineffective if delayed.
BAL (prophylaxis)	40	1	3	2	5.0 ± 8.4	BAL deleterious as prophylactic.
BAL intraperit. and intramusc.	40	3 to 6	12	69	57.9 ± 4.4	Oral administration seems least effective, but differences of doubtful significance.
BAL intramusc. only	40	2 or more	19	59	49.6 ± 4.2	
BAL oral only	40	4	4	55	45.8 ± 6.5	

N.B. Although this analysis was made before all the data were available, it reflects the later data also.

to more and to earlier deaths, presumably because BAL fixes 2 to 3 times as much Cd in the lungs as would otherwise remain there (17).

BAL is rather less effective in treating dogs poisoned by inhaled $CdCl_2$. Used near its tolerated maximum dose (30 mg. per kg. every 3 hours for four doses), dogs were worse with BAL than without; but when used in a lower dose (12.5 mg. per kg. repeated 6 to 10 times at 2 hour intervals) BAL improved survival. Of

19 dogs given BAL, 65 per cent lived over two weeks and 42 per cent lived indefinitely (over 6 weeks), as compared with 32 untreated animals, all of which died, mostly in 3 to 5 days. Further, the BAL-treated dogs which did die survived, on the average, twice as long as the untreated animals. Comparable results on dogs have been obtained by others (25).

Effect of BAL on body distribution of cadmium. The organ distribution of Cd, administered with and without treatment, has been followed by using the radioactive isotope and more or less standard tracer technique (17). Over half of the inhaled $CdCl_2$ has passed beyond the lung by the end of a 30-minute exposure period. Cd continues to leave more and more slowly for about two days, when half the initially retained amount has left. The curve of per cent loss is similar for all initial lung concentrations. When CdS dust is inhaled, however, the

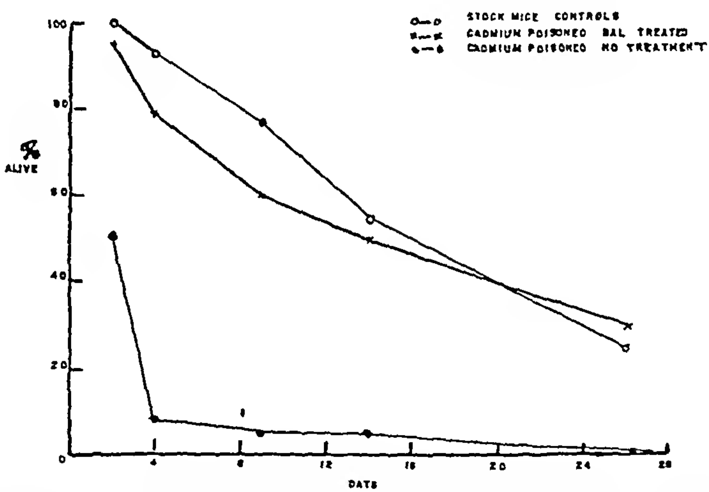


Fig. 2. Effect of Spontaneous Death Rate in Unhealthy Stock Mice on Apparent Therapeutic Effect of BAL

insoluble particles leave the lung extremely slowly. If BAL is present at the time of Cd inhalation, the Cd is fixed in the lung, presumably as insoluble Cd-BAL, and over twice the absolute amount of Cd is found there at the end of exposure or at any subsequent period. This is associated with increased lung damage. BAL given after Cd exposure, despite the fact that it materially decreases lung damage, neither accelerates nor delays Cd loss from the lung. Prophylactically administered BAL apparently does damage by holding a large amount of Cd for slow release to lung tissue, while therapeutically administered BAL, reaching the lung after much of the Cd has left, diverts Cd already combined with lung tissue constituents and then releases it slowly enough so that most is removed. The mechanism of BAL action on lung Cd content and pathology deserves further study.

In the untreated animal, most of the Cd that passes from the lung and is not fixed elsewhere eventually leaves the body via the gastro-intestinal tract. Little is excreted through the kidneys. Treatment with BAL, however, definitely shifts excretion in the direction of the kidney. This is reflected in a changed pathological picture.

Effect of BAL on structural pathology. BAL alters the pathological changes produced in mice by inhaled Cd, as shown in figure 1. Perivascular, peribronchiolar edema still occurs, but to only about half the extent seen in untreated animals, and alveolar edema occurs minimally if at all. Edema is maximal by

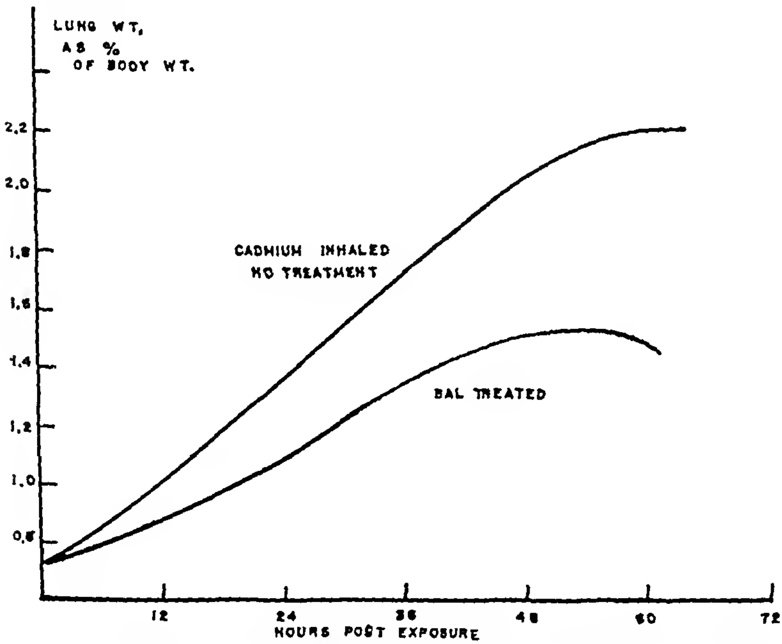


FIG. 3. LUNG WEIGHT, BODY WEIGHT RATIO IN TREATED AND UNTREATED MICE AFTER CADMIUM INHALATION

about 36 hours and then regresses, until at 100 hours after exposure no sign of it remains. This sharp contrast to untreated animals (edema maximum at 50 hours and not subsided by 100) is reflected in the ratio of lung weight to body weight (fig. 3), and the more normal appearance of the treated lungs at all times after exposure (fig. 4).

The inflammatory response also differs from that in untreated animals, being less severe and diffuse. It remains localized mainly to the peribronchiolar, perivascular connective tissue and mucosa. The adjacent alveoli and atria are infiltrated and contain polymorphonuclear leucocytes and macrophages. About

two days after exposure the leucocytes begin to disappear and macrophages become more prominent. Resolution takes place rapidly but is not complete, since fibroplasia and fibrosis of these regions do occur. The scarring is much less severe, however, than in the untreated cases. Many animals, both mice and dogs, develop secondary suppurative bronchopneumonia, bronchitis and lung

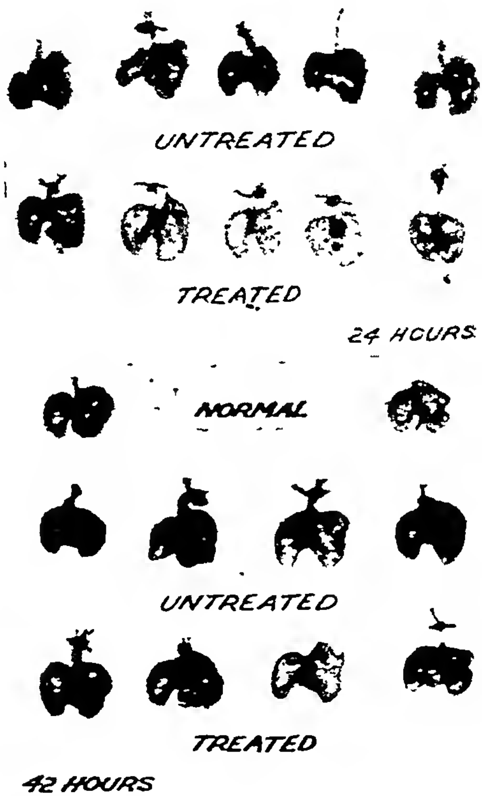


FIG. 4. GROSS APPEARANCE—CD-POISONED MOUSE-LUNGS, WITH AND WITHOUT BAL

abscesses, and these lesions are probably the main cause of late deaths. Proper anti-bacterial chemotherapy would almost surely decrease these.

The reactive changes in the jejunal and gastric mucosae of mice, produced by BAL, alone or after Cd inhalation, appear earlier and are less intense and enduring than those seen after untreated Cd poisoning. In BAL-treated, Cd-poisoned mice, thymic lymphorrhexis is minimal, but some atrophy of the gland takes place. Regeneration starts about 80 hours after treatment is begun.

BAL treatment of dogs poisoned with intraperitoneal Cd produces profound liver changes. The sinusoids become jammed with swollen Kuppfer cells, polymorphonuclear leucocytes and many immature cells. Intravenous Cd followed by intravenous BAL produces intense renal changes in dogs. Epithelial cell nuclei in the distal convoluted tubules, Henle's loop and the upper part of the collecting tubules are greatly enlarged and prominently nucleolated. Multinucleated cells and mitotic figures are common, and the tubules contain hyaline and granular casts. The glomeruli appear normal except for some infiltration with polymorphonuclear leucocytes. Kidney damage under these conditions has also been seen by others (9). No renal pathology has been observed following BAL treatment of poisoning by inhaled Cd, although BAL does increase the Cd content of kidney and urine as well as of the gastrointestinal tract (17).

TABLE 7

Toxicity of thiols—mice

NO. OF ANIMALS	COMPOUND	VEHICLE	ROUTE	TOLERATD SINGLE DOSE		TOLERATED REPEATED DOSE mM/kg.		TOLERATED REPEATED DOSE Number	TOLERATED REPEATED DOSE Interval
				mM/kg.					*hrs.*
	BAL	Saline	Intramusc.	0.4		0.16		6–8	2
4	Cysteine hydrochloride	Saline	Intraperit.	12					
5	Ethanedithiol	Saline	Intramusc.			0.05		6	2
5	1,3-dithioglycerol	Saline	Intramusc.			0.05		6	2
5	Propanedithiol-1,2	Saline—acacia	Intramusc.			0.1		5	2
5	Propanedithiol-1,3	Saline—acacia	Intramusc.			0.20		5	2
10	BAL sarcosine methylene mercaptid	Saline	Intramusc.	0.8					
5	BAL glycine methylene mercaptid	Saline	Intramusc.	1.5		0.43		9	In 73 hrs.
10	2,3-dimercapto propyl acetate	Saline—acacia	Intramusc.			0.1		6	2
5	2,3-dimercapto propyl-ethyl ether	Saline	Intramusc.	<0.4					
15		Peanut oil	Intramusc.			0.2		8	2
19		Undiluted	Percutan.	<0.4		0.2		4	2
5	n-(2,3-dimercapto propyl) carbamate	Saline—acacia	Intramusc.			0.05		5	2
10	Bis-S(N-ethylacetamidomethyl) ether of BAL	Saline	Intramusc.	0.96		0.32		6	2
				mg/l	*mins*	*mg/l*	*mins*		
9	HSH		Inhaled	1.5	20	0.4	30	4	2
7	Ethyl mercaptan		Inhaled	54	10	4.5	30	5	2

Other thiols. Many thiols were prepared (25) as potential therapeutic agents,

and some fifteen have been tested for toxicity and therapeutic efficacy (tables 7 and 8). In summary, none was demonstrated to be as effective as BAL, but none received comparably exhaustive study. Several combined BAL compounds

TABLE 8

Treatment of mice poisoned by inhaled cadmium chloride with agents other than BAL*

AGENT	TREATMENT				PER CENT MORTALITY AT DAYS INDICATED†					
	Dose	No. of doses	Dose interval	Route	4 Controls	4 Treated	9 Controls	9 Treated	14 Controls	14 Treated
	ml/kg		*hrs.*							
BAL (for comparison).	0.16	6	2	Intramusc.	93	7	93	7	93	7
BAL glycine methylene mercaptid.	0.32	6	2	Intramusc.	70	0	88	13	88	13
bis-S(n-ethylacetamidomethyl) ether of BAL	0.16	6	2	Intramusc.	70	7	88	7	88	20
2,3-dimercapto propyl ethyl ether	0.20	6	2	Intramusc.	100	34	100	60	100	87
2,3-dimercapto propyl acetate	0.10	1		Intramusc.	93	53	93	80	100	93
1,3-dithioglycerol	0.05	6	2	Intramusc.	93	80	93	93	93	100
Ethanedithiol	0.05	6	2	Intramusc.	93	80	93	100	93	100
BAL sarcosine methylene mercaptid	0.40	1		Intramusc.	80	93	86	100	86	100
Selenium dioxide	4.8×10^{-4}	1	Prophylaxis	Intraperit.	87	40	100	53	100	53
Hexamethylenetetramine	140	1	Prophylaxis	Intraperit.	93	93	93	93	93	93
Sodium formaldehyde sulfoxalate	6.5	1	Prophylaxis	Intraperit	73	73	73	73	73	73
Sodium thiosulphate..	8.1	1	Prophylaxis	Intraperit.	100	100	100	100	100	100
Hexamethylenetetramine alliodide	1.0	1	Prophylaxis	Intraperit.	73	80	73	80	73	80
4 methyl-1,2 naphthoquinone	0.3	1	Prophylaxis	Intraperit.	93	93	93	93	93	93
	mg./l. *mins.*									
Ethyl mercaptan	9 10	1	Immediate	Inhalation	86	47	93	66	100	66
	4.5 30	4, 3, 1	2 × on 1st, 2nd, 3rd days							

* Exposed to 0 05 to 0.19 mg. Cd. per l. (as $CdCl_2$) for 20 to 40 mins.
† Average number of mice per group, 15.

—bis-S(n-ethylacetamido-methyl) ether of BAL, BAL sarcosine-methylene-mercaptid, and BAL glycine-methylene-mercaptid—were only one-quarter to one-half as toxic for mice, on a molar basis, as BAL: and the first and last of these gave highly encouraging results in treating mice poisoned by Cd inhalation.

Encouraging results were also obtained with 2,3-dimercapto-propyl-acetate and 2,3-dimercapto-propyl-ethyl-ether. With optimal dose, time and frequency of administration, some of these compounds might prove superior to BAL. Ethyl mercaptan administered by inhalation (9 mg. per liter for 30 mins. immediately after exposure, and 4.5 mg. per liter for 30 mins. repeated several times over 2 to 3 days) also gave encouraging therapeutic results (table 8).

Selenium. The therapeutic effect of selenium dioxide was tested (table 8) on the basis of a previously observed Se-As antagonism, even though no Se-Cd antagonism was seen (13). The formation of Se analogue of cysteine has been postulated (13). This could then combine with As, or Cd, and both metals be excreted as an As-Se-mercaptide. In Cd poisoned mice, Se did have a therapeutic effect and, unlike the thiols, could also be used prophylactically (table 8). One intraperitoneal injection of 3 mg. Se per kg. (as SeO_2) before and after exposure, or an injection of 1.5 mg. per kg. on the first and second days after exposure, saved about 40 per cent of mice which would otherwise have succumbed. Optimal dosage and route of administration were not determined.

SUMMARY

Animals fatally poisoned by the inhalation of Cd compounds die early (hours to a few days) with massive pulmonary edema and symptoms mainly referable to anoxic anoxia; later deaths (a few days to a week or so) occur as a result of diffuse pneumonia, sometimes with lung abscess formation; long delayed deaths (several weeks to a month) occur with incompletely healed pulmonary lesions (mainly secondary infection), anorexia, occasional bloody diarrhoea and generalized wasting. Symptoms referable to gastro-intestinal damage are common in all.

Structural damage after Cd inhalation is most marked in the lungs, consisting chiefly of early edema followed by later cellular infiltration, generalized pneumonitis, bronchiolitis, bronchitis and often mucosal sloughing, and still later by healing and fibrosis. Epithelial cell reactive changes and increased mitoses are seen in the gastric and intestinal mucosae; lymphorrhexis in the thymus. Other organs show no consistent change except for marked Kuppfer cell swelling after intraperitoneally injected Cd.

The dithiol BAL (2,3-dimercaptopropanol) is therapeutically effective in Cd poisoning, but is deleterious if given prophylactically for poisoning produced by inhalation. Injected promptly after exposure it can, in an optimal course of repeated injections, reduce mortality from 93 to 7 per cent. It greatly ameliorates the structural damage and markedly alters body distribution and route of excretion of Cd (lungs, kidneys, gastro-intestinal tract), as followed with the radioactive isotope Cd^{115}. In mice and dogs, BAL, to be effective, must be used in amounts near the tolerated maximum. The optimal and maximum tolerated doses, for single and repeated administration have been studied, and a standard course of treatment for mice, based on a study of 2500 animals, is described.

A number of non-thiols and thiols other than BAL were examined, though less extensively, as potential therapeutic agents. Several are effective and further study might reveal some to be superior to BAL. One of these substances, sele-

nium, is also effective prophylactically as well as therapeutically. The use of selenium merits further exploration.

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THE EFFICACY OF 2,3-DIMERCAPTOPROPANOL (BAL) IN THE THERAPY OF POISONING BY COMPOUNDS OF ANTIMONY, BISMUTH, CHROMIUM, MERCURY AND NICKEL

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Recent investigations of the biochemistry of the dithiol BAL by Barron and Kalnitsky (1) indicate that arsenicals interfere with tissue respiration by inhibiting the action of a large group of SH enzymes involved in carbohydrate and fat metabolism. These investigators working with the SH-containing enzyme succinoxidase showed that in vitro the toxicity of such heavy metals as Pb, Sb, V, Bi, Cd, Hg and Zn is due, as in the case of As, to the inhibition of the SH enzymes. BAL and some related compounds reversed these inhibitions. In view of this concept, it is of interest to determine whether BAL is of therapeutic value in the treatment of heavy-metal poisoning in the intact animal.

The successful treatment of Lewisite burns with BAL led us to investigate the therapeutic effectiveness of this dithiol in acute intoxications caused by compounds of the following metals: Sb, Bi, Ni, Cr, Hg, Pb, Tl and Se.

Procedure. Rabbits weighing between 2000 and 3000 gms. were selected. The animals were obtained from several sources and consequently could be expected to give a heterogeneous response to the toxic substances. The sexes were approximately equally distributed.

In our experiments, control and treated groups of animals were run in parallel. In the control animals, a single toxic dose of the metallic compound was injected intramuscularly into the gluteal muscles of the right leg. The treated animals received a similar injection of the metallic compound and in addition were given a freshly prepared, 3 per cent, aqueous solution of BAL by injection into the gluteal muscles of the left leg according to the following schedule. A dose of 30 mg./kg. of BAL was administered one hour after the administration of the toxic substance; doses of 15 mg./kg. of BAL were administered six, twenty-four and forty-eight hours thereafter. There were certain exceptions to this course of treatment as will be noted below. The animals were kept under observation for 30 days and all deaths within this period were included in the results.

BAL is not an innocuous compound. When injected intramuscularly in the rabbit it has an LD50 of 99 mg./kg. In a series of subacute experiments in which BAL was administered intramuscularly daily for 12 days, we have determined that the rabbit will tolerate a daily dose of 10 mg./kg. without manifesting toxic symptoms. At a daily dose of 15 mg./kg., one of 10 animals died on the twelfth

[1] A portion of the funds used in this investigation was supplied by a transfer, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Division of Pharmacology of the Food and Drug Administration.

day and the majority of the rabbits showed losses of weight amounting to as much as 300 gms.

Dosage-mortality curves were constructed in all cases according to the method of Bliss (2). The LD50 for the control animals was selected as the most useful measure of the toxicity of the compounds under study, and the success of treatment was judged in each case by the extent to which the LD50 for BAL-treated animals departed from this value.

EXPERIMENTAL. *Antimony.* Antimony compounds have been extensively employed during the war especially in the tropics where they were used for the treatment of leishmaniasis, filariasis and schistosomiasis. Antimony poisoning may be readily produced by the improper medication with any of the available antimonials.

In our investigations of the protective action of BAL in acute antimony poisoning, 3 compounds were utilized, namely, an organic antimony salt—antimony tartrate, a trivalent organic antimony compound—Fuadin, (Sodium antimony biscatechol disulfonate of sodium), and a pentavalent compound—Neostam (nitrogen glucoside of sodium p-aminophenylstibonate).

The control animals showed no striking symptoms but became apathetic. When death occurred in either the control of treated rabbits, it was in from two to six days. Animals surviving this period recovered completely. The data in table 1 demonstrate the protective action of BAL against acutely toxic doses of compounds of antimony. With the administration of BAL, the tolerance of the rabbits to lethal doses of compounds of antimony was increased more than 50 per cent. BAL is effective not only against organic salts of antimony but also against organic trivalent and pentavalent antimony compounds.

Bismuth. In order to evaluate BAL as treatment in acute bismuth poisoning, a water-soluble salt sodium, potassium bismutho tartrate was utilized. It is an established fact that water-soluble bismuth salts are rapidly absorbed and consequently they are the most toxic group of bismuth salts used medicinally. Absorption occurs rapidly from the site of injection and after 10 days we were able to detect only traces of the metal in the gluteal muscles. Death, when it occurred, was in from two to nineteen days with the greatest mortality from the second to the sixth day. The control rabbits displayed a loss of appetite with a consequent loss of weight and died in coma with the usual uremic symptoms.

We were unable to detect any marked increase in either urinary volume or total bismuth excretion in the treated animals. However, BAL did protect the kidneys of the treated animals. At autopsy, the kidneys of the animals of the control group were severely damaged as shown by marked enlargement, a pale grayish brown color, and grittiness when cut. Grossly, the kidneys of the treated animals were essentially normal. This was confirmed by microscopic sections in several instances. At the toxic level of 70 mg./kg. of sodium potassium bismutho tartrate, the total percentage output of bismuth excreted over 12 days as determined by Leonard's method (3) was greater than that excreted at the subtoxic level of 50 mg./kg.

The protection afforded by treatment with BAL in rabbits acutely poisoned with bismuth is demonstrated in table 2. The LD50 for the control group is 55 mg./kg. compared with an LD50 of 85 mg./kg. for the treated animals.

Chromium. In our studies to evaluate the efficacy of BAL in the treatment of heavy metal poisoning, we have found it to be an effective treatment for lethal

TABLE 1

Effect of BAL on acute antimony poisoning in rabbits

COMPOUND	MET Sb mg./kg	CONTROL			TREATED		
		No of animals	No survived	Per cent survived	No of animals	No survived	Per cent survived
Antimony tartrate							
mg./kg							
50	15.30	5	5	100			
60	18 4	6	5	83			
75	23 0	8	3	38			
100	30 6	11	3	27			
125	38 3	8	2	25	15	12	80
150	45.9	11	0	0	14	7	50
175	53.6	5	0	0	12	6	50
200	61.3	5	0	0	15	7	47
		LD50 = 90 mg./kg			LD50 = 160 mg./kg.		
Fuadin							
118	10.0	12	1	8	16	15	94
148	12.5	4	0	0	3	3	100
177	15.0	5	0	0	7	4	58
206	17.5	2	0	0	8	3	38
235	20.0	5	0	0	25	5	20
		LD50 = 91 mg./kg.			LD50 = 162 mg./kg.		
Neostam							
50	12.1	3	3	100			
100	24.2	11	4	36	10	9	90
150	36 2	11	5	45	10	6	60
175	42 3	11	1	9	10	5	50
200	48 3	6	0	0	12	3	25
225	54.4	6	0	0	12	3	25
250	60 4	3	0	0	12	3	25
		LD50 = 91 mg./kg			LD50 = 174 mg./kg.		

doses of chromates in rabbits. Chromium compounds have long been regarded as an industrial hazard in the mining of chrome ores as well as in those branches of industry utilizing chromates, namely, in dyeing, plating and electrotyping.

Potassium chromate was used to produce the acute chromium poisoning. Due to the severe nephrotoxic action of this salt, it was necessary to modify the pro-

cedure used heretofore. Treatment with BAL was continued over a period of 9 days. During the last 6 days, 7½ mg. of BAL/kg. was administered daily.

Treatment with BAL must be started within 1 hour after the administration of the potassium chromate if it is to be therapeutically effective. Animals receiving the chromate showed anorexia, loss of weight, and died in a coma. Death occurred in from two to eight days after the administration of the potassium chromate.

The data in table 3 would indicate that in rabbits treated with BAL the fatal

TABLE 2

Effectiveness of BAL in the treatment of acute bismuth poisoning in rabbits

SALT	MET. Bi	CONTROL			TREATED		
		No. of animals	No. survived	Per cent survived	No. of animals	No. survived	Per cent survived
mg./kg.	*mg./kg.*						
50	20.5	7	6	88	6	6	100
60	24.6	8	1	13	4	4	100
70	28.5	7	0	0	8	7	88
80	32.8	6	0	0	10	7	70
90	36.9	6	0	0	10	5	50
100	41.0	12	0	0	16	2	13
110	45.1				5	0	0
		LD_{50} = 55 mg./kg.			LD_{50} = 85 mg./kg.		

TABLE 3

Effectiveness of BAL in the treatment of acute chromate poisoning in rabbits

SALT	MET. Cr	CONTROL			TREATED		
		No. of animals	No. survived	Per cent survived	No. of animals	No. survived	Per cent survived
mg./kg.	*mg./kg.*						
10.0	2.7	3	3	100			
12.5	3.4	5	3	60	5	4	80
15.0	4.0	8	0	0	23	12	52
17.5	4.7	5	0	0	8	3	38
20.0	5.4	13	0	0	8	2	25
25.0	6.7	4	0	0	12	0	0
		LD_{50} = 10.8 mg./kg.			LD_{50} = 16 mg./kg.		

dose of potassium chromate is about one and one-half times that for untreated rabbits.

Nickel. Although salts of nickel are relatively unimportant from a therapeutic and toxicological standpoint, the effectiveness of BAL in combatting acute nickel intoxication was studied. Eighty rabbits, both control and treated animals, were injected with varying doses of nickel chloride. Death supervened in from one to fourteen days, most frequently between the third and eighth days.

It would appear from the results shown in table 4 that the tolerance of BAL-

treated rabbits to which lethal doses of nickel chloride were administered is increased more than 90 per cent.

Mercury. Gilman, Allen and Philips (1) have reported the effectiveness of BAL as an antidote for bichloride of mercury poisoning. Longcope and Leutscher (1) have demonstrated this effect clinically. The evidence presented by us confirms the findings of Gilman and coworkers.

As in the case of chromium poisoning, it was found necessary to modify our procedure by initiating a 9-day course of treatment. During the last 6 days, a

TABLE 4

Effectiveness of BAL in the treatment of acute nickel poisoning in rabbits

SALT	MET. Ni	CONTROL			TREATED		
		No. of animals	No survived	Per cent survived	No. of animals	No survived	Per cent survived
mg./kg.	mg./kg.						
20	9.0	6	5	84			
30	13.6	6	3	50			
40	18.1	8	0	0	10	9	90
50	22.6	10	0	0	10	6	60
60	27.1	4	0	0	8	2	25
75	34.0	10	0	0	8	0	0
		LD_{50} = 27 mg./kg.			LD_{50} = 52.5 mg./kg.		

TABLE 5

Effectiveness of BAL in the treatment of acte mercury poisoning in rabbits

SALT	MET Hg	CONTROL			TREATED		
		No. of animals	No survived	Per cent survived	No of animals	No. survived	Per cent survived
mg./kg.	mg./kg.						
8	5.9	6	4	33			
9	6 7	5	1	20	6	6	100
10	7.4	5	0	0	11	8	73
12	8.9	6	0	0	10	3	30
15	11.1	6	0	0	5	0	0
		LD_{50} = 7.3 mg./kg.			LD_{50} = 11.75 mg./kg.		

daily dose of 7½ mg./kg. of freshly prepared aqueous BAL was administered. Treatment with BAL must be promptly started after the administration of bichloride of mercury. We were unable to save animals after more than one hour had elapsed before treatment was instituted. Necrosis and dry gangrene were apparent distal to the site of injection in the control animals but this was not true in the case of animals treated with BAL.

Lead. Our investigations have led us to the conclusion that BAL is not an effective treatment of either acute or chronic lead poisoning. In the acute experiments, a single dose of lead nitrate was injected intraperitoneally and treatments

with BAL (10 mg./kg. daily) were administered intramuscularly for 10 days. Death occurred in from two to twenty-one days with the greatest mortality from the fifth to the ninth day. The data indicated that the treated animals were more susceptible than the controls.

Twenty-four rabbits were used in the chronic experiment. Fifteen mg./kg. of lead nitrate injected intraperitoneally was administered daily for 14 days. Following the completion of these injections, 7 days were permitted to elapse before treatment with BAL was initiated. The series was divided into 3 groups of 8 rabbits per group. One group of the animals received daily intramuscular injections of 6 mg./kg. of BAL while to a second group a daily dose of 10 mg./kg. was administered by the same route of administration. The third group was composed of the control animals which received no BAL. Within the 30-day period of the experiment, all of the treated animals died while all the control animals survived. These experiments would tend to indicate that in both acute and chronic lead poisoning BAL has an additive effect.

Thallium. BAL has no therapeutic efficacy in the treatment of acute thallium intoxication. In this investigation 74 rabbits were utilized at the various dosage

TABLE 6

Effectiveness of BAL in the treatment of acute lead poisoning in rabbits

SALT	MET. Pb	CONTROL			TREATED		
		No. of animals	No. survived	Per cent survived	No. of animals	No. survived	Per cent survived
mg./kg.	*mg./kg.*						
80	50.1	9	4	44	10	2	20
90	56.3	9	3	33	10	0	0
100	62.6	9	0	0	7	0	0

levels of the control and treated series. At the toxic doses of thallium sulphate, both the control and BAL-treated rabbits died in from 3 to 9 days. Daily administration of BAL to the treated animals was continued for the duration of the experiment. A comparison of the average number of days of survival yielded no significant difference between the control and treated series.

Selenium. The ineffectiveness of BAL as a treatment for selenium poisoning was demonstrated in both acute and subacute experiments. Sodium selenite was used as the source of the selenium. It soon became apparent in the acute experiments, in which 84 rabbits were used, that animals treated with BAL at toxic levels of Se were more susceptible than the controls. Animals receiving 0.8 mg./kg. of Se plus the BAL died in from two to six hours, while those of the control series which received the selenium but not the BAL lived several days.

This additive effect was even more marked in the subacute experiment. Of 16 rabbits, selected for this 30-day experiment, 8 served as controls and received intramuscular injections of 0.3 mg./kg. of Se daily. The remaining animals composed the treated series and received in addition to the 0.3 mg./kg. of Se 10

mg./kg. of BAL per day. All the animals of the control series survived the duration of the experiment and gained from 200 to 800 gms. in weight. Six of the 8 animals of the treated series died in from ten to fourteen days. The 2 surviving animals lost 300 and 400 gms. of weight respectively.

SUMMARY AND CONCLUSIONS

1. The effectiveness of BAL as a treatment in heavy-metal poisoning was investigated on 1226 rabbits.

2. BAL was found to be an effective antidote in acute poisoning caused by the administration of toxic doses of salts of antimony, bismuth, chromium, nickel and mercury in the rabbit. With the administration of BAL the tolerance of rabbits to lethal doses of compounds of these heavy metals was increased by at least 50 per cent.

3. BAL was ineffective in the treatment of rabbits acutely poisoned by salts of lead, thallium and selenium. In the case of lead and selenium, the action was additive.

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